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Trondheim, June 2017

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Norsk sammendrag:

Humant metapneumovirus: Kliniske og virologiske aspekter hos norske barn

Humant metapneumovirus (HMPV) ble oppdaget i 2001. Senere studier har vist at HMPV er likt et annet virus, som heter respiratorisk syncytialt virus (RSV), og at viruset ofte fører til luftveisinfeksjon (LVI) hos barn. Formålet med dette prosjektet har vært å se nærmere på forekomst og kliniske funn ved alvorlige HMPV-infeksjoner hos norske barn, samt betydningen av ulike HMPV-typer og samtidig tilstedeværelse av andre virus. Vi ønsket også å sammenligne alvorlige HMPV- og RSV-infeksjoner. I tillegg ønsket vi å finne ut hvor lang tid HMPV finnes i luftveissekret i forbindelse med en infeksjon, og om HMPV finnes hos barn som er uten luftveisinfeksjon (friske sykehuskontroller). Dette gjorde vi for å undersøke nærmere om utskillelsestiden for HMPV eventuelt kan forklare forekomsten av HMPV hos barn uten infeksjon. Vi ønsket også å undersøke forekomsten av HMPV og andre luftveisvirus blant barn som går i barnehage.

Materialet til disse undersøkelsene baserte seg på 3 kliniske studier, hvor den ene inkluderte 3,650 barn fra sykehus med luftveisinfeksjon og 339 friske sykehuskontroller, den andre inkluderte 32 barn innlagt med HMPV-infeksjon for å undersøke utskillelsestiden, og den tredje inkluderte 161 barn fra to barnehager. Barna i barnehagene deltok i gjennomsnitt 2 ganger, da vi gjennomførte 4 besøk i løpet av 2 år. Det ble innhentet skriftlig samtykke fra foresatte på vegne av barna for deltagelse i studiene. De aller fleste barna ble klinisk undersøkt og det ble tatt sekret-prøver fra bakre området av nese og svelg. Disse sekret-prøvene ble undersøkt med polymerase kjedereaksjon-tester (PCR) for påvisning av HMPV, RSV og 17 andre luftveispatogener, samt dyrket i cellekulturer. De prøvene som var positive for HMPV ble typet, og det ble gjort fylogenetiske analyser.

HMPV ble påvist hos 7.3% og RSV hos 28.7% blant barn på sykehuset i løpet av perioden 2006-2015. Blant de friske sykehuskontrollene var det 2.1% som fikk påvist HMPV i lav virusmengde ved PCR-test, men alle var negative på dyrkningen. HMPV opptrådte i regelmessige epidemier i løpet av flere vintre, men også under vår-sommer perioder. Epidemiene varte i gjennomsnitt 3.5 måneder. Blant barn <5 år gamle, ble det beregnet at HMPV forårsaket årlig sykehusinnleggelse med nedre LVI hos 1.8/1000 barn, mens RSV medførte sykehusinnleggelse hos 9.9/1000 barn. Alle de ulike typene av HMPV (A2a, A2b,

B1 og B2), med unntak av A1, ble påvist. I løpet av hvert år sirkulerte 2 eller flere typer HMPV, og de fylogenetiske undersøkelsene viste ingen spesielle grupperinger eller nye typer i løpet av hele perioden. Grupper av barn med alvorlige HMPV-infeksjoner hadde helt like kliniske funn ved ulike HMPV-typer og om andre virus var til stede eller ikke. Barn som hadde alvorlige HMPV- og RSV-infeksjoner hadde også nokså like kliniske funn. Nærmere undersøkelser viste at barnets alder, samt det å ha vært for tidlig født og ha en kronisk sykdom, var assosiert med høy risiko for alvorlige HMPV- og RSV-infeksjoner. Blant barn som var <6 måneder gamle var det sjelden at HMPV-infeksjon medførte alvorlig sykdom, men det gjorde derimot RSV. Mens blant barn i aldergruppen 12-23 måneder medførte HMPV oftere mer alvorlig sykdom enn RSV. Blant barn i barnehagene, fant vi HMPV bare 4 ganger, mens rhinovirus, enterovirus og parechovirus var hyppige. Forekomsten av luftveisvirus hos barn i barnehagene varierte med barnets alder, tegn til luftveisinfeksjon ved klinisk undersøkelse, avdeling (avdeling for yngre barn eller eldre barn) og årstid. Utskillelsestiden av HMPV ble beregnet til å være median 13 dager (variasjonsbredde 6-28 dager).

HMPV forekom i regelmessige epidemier, og viruset er ofte en årsak til alvorlige luftveisinfeksjoner hos norske barn, selv om den årlige innleggelsesraten på sykehus er lavere for HMPV enn RSV. De kliniske funn ved alvorlige HMPV- og RSV-infeksjoner er ganske like, og de kliniske funn ved alvorlige HMPV-infeksjoner påvirkes ikke av HMPV-typer eller om andre virus er til stede. Utskillelsestiden av HMPV er kort, HMPV påvises sjelden hos friske sykehuskontroll-barn og barnehagebarn, mens ulike picornavirus kan hyppig påvises hos norske barnehagebarn.

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Human metapneumovirus: Clinical and virological aspects in Norwegian children

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List of papers

Paper I

The Burden of Human Metapneumovirus Infections in Hospitalized Norwegian Children

Moe N, Stenseng IH, Krokstad S Christensen A, Skanke LH, Risnes KR, Nordbø SA, Døllner H.

Submitted manuscript*

Paper II

Comparing Human Metapneumovirus and Respiratory Syncytial Virus: Viral Co-detections, Genotypes and Risk Factors for Severe Disease

Moe N, Krokstad S, Stenseng IH, Christensen A, Skanke LH, Risnes KR, Nordbø SA, Døllner H.

PLoS One. 2017; 12:e0170200, Epub 2017/01/17

Paper III

Respiratory Virus Detection and Clinical Diagnosis in Children Attending Day Care

Moe N, Pedersen B, Nordbø SA, Skanke LH, Krokstad S, Smyrnaios A, Døllner H.

PLoS One. 2016; 11(7):e0159196, Epub 2016/07/21

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Abbreviations

AMPV	Avian metapneumovirus
CAIR	Childhood Airway Infections Research Group
CI	Confidence intervals
CRP	C-reactive protein
Ct value	Cycle threshold value
DP	Department of Pediatrics
ELISA	Enzyme linked immunosorbent assay
GLMM	Generalized linear mixed-effect models
HAdV	Human adenovirus
HBoV	Human bocavirus
HCoV	Human coronavirus
HEV	Human enterovirus
HMPV	Human metapneumovirus
HPeV	Human parechovirus
HRV	Human rhinovirus
IF	Immunofluorescence
LRTI	Lower respiratory tract infection
NPA	Nasopharyngeal aspirates
NPS	Nasopharyngeal samples
OR	Odds ratios
PCR	Polymerase chain reaction
PICU	Pediatric intensive care unit
PIV	Parainfluenza virus
RSV	Respiratory syncytial virus
RTI	Respiratory tract infection
URTI	Upper respiratory tract infection

Summary

Background

Human metapneumovirus (HMPV) was discovered in 2001. Later studies have shown that HMPV is quite similar to respiratory syncytial virus (RSV), and a common cause of respiratory tract infections (RTI) in children.

Aims

The aims of my thesis were to study the occurrence and clinical manifestations of severe HMPV infections in Norwegian children and the impact of viral co-detections and HMPV genotypes. I also wanted to compare severe HMPV and RSV infections. I wanted to study the HMPV shedding time during acute RTI and HMPV in asymptomatic hospital controls, to get an impression of HMPV in non-infected children. Finally, I wanted to study HMPV and other respiratory viruses among children in day-care centers.

Materials and Methods

The thesis is based on data from 3 clinical studies, a cohort study of 3,650 hospitalized children with RTI and 339 asymptomatic hospital controls, the shedding study including 32 hospitalized HMPV-infected children and a cross-sectional study in two day-care centers, including 161 apparently healthy children. The day-care children were examined median 2 times during four visits in 2012-2014. Informed, written consents to participate were collected from caregivers and the majority of children underwent clinical examination. Nasopharyngeal samples were analyzed by polymerase chain reaction (PCR) tests for HMPV, RSV and 17 other pathogens, and cultured for virus. HMPV-positive samples were genotyped and phylogenetic analyses were performed.

Results

HMPV was detected in 7.3% and RSV in 28.7% among children in the hospital cohort during the period 2006-2015. Among the controls, 2.1% had HMPV with low viral load by PCR, but all were culture negative. HMPV occurred in regular winter and spring-summer epidemics, and with a median duration of 3.5 months. The average annual hospitalization rates in children <5 years old with lower RTI were 1.8/1,000 (HMPV) and 9.9/1,000 (RSV). All HMPV subtypes (A2a, A2b, B1 and B2), except subtype A1, were detected. In each season, at least two subtypes circulated. The F gene sequencing revealed no clusters or new strains, whereas several known HMPV strains circulated during the entire period. The clinical manifestations were relatively similar in HMPV- and RSV-infected children with lower RTI, and the clinical manifestations in HMPV lower RTI were not related to viral co-detection and

HMPV genotypes. Age was an important risk factor for disease severity, in addition to a history of prematurity and chronic disease, for HMPV- and RSV-associated lower RTI. Children <6 months of age with HMPV had a milder disease than those with RSV, while in children 12-23 months old, the pattern was the opposite. Among children in day-care centers, HMPV was detected 4 times only, but rhinovirus, enterovirus and parechovirus were frequently appearing. The virus rates in day-care children varied in relation to age, clinical signs of RTI, day-care section (sections for younger and older children) and season. The median HMPV shedding time was estimated to be 13 days (range 6-28 days).

Conclusions

HMPV occurred in regular epidemics, and is a common cause of severe RTI in Norwegian children, but the hospitalization rate is five times lower than RSV. Clinically, severe HMPV and RSV infections manifest relatively similar, and independent of viral co-detections and HMPV genotypes in children with severe HMPV infections. Children shed HMPV shortly and HMPV appears seldom in asymptomatic children and healthy day-care children, in contrast to picornaviruses that may be detected frequently in Norwegian children attending day care.

1 Introduction

Human metapneumovirus (HMPV) was discovered in respiratory specimens from young children with respiratory tract infection (RTI) in 2001 (1). Later studies have shown that HMPV is an epidemic virus that occurs in outbreaks worldwide (2-7). As early as in 2003, our research group reported the first HMPV detection in Norway (8), and in 2004 they reported an outbreak of HMPV among hospitalized children, where more than 50% of all children during a short period had HMPV (9). An important background for understanding the discovery of HMPV and several other newly identified pathogens during the last decade, is the development of new nucleic acid-based methods (1, 10). In recent years, the use of these sensitive methods has contributed significantly to the increased detection rates of respiratory viruses (10). Additionally, viral co-detection of two or more viruses from each child has been shown to occur frequently (5, 11). HMPV is classified in *Pneumoviridae* family with respiratory syncytial virus (RSV), and the causal role of HMPV as a respiratory pathogen has been confirmed in both clinical (1, 2, 12, 13) and in experimental studies (14, 15).

In this thesis, I have focused on severe HMPV infections in Norwegian children. I have also tried to get an impression of HMPV among asymptomatic children and apparently healthy children, by studying a group of hospital controls and children in two day-care centers. In the studies, I wanted to establish the burden of severe HMPV infections defined as hospitalized children with RTI. In the analyses, I have compared with RSV, the most common respiratory virus causing severe RTI, and I have considered the impact of viral co-detections and HMPV genotypes. I have examined the HMPV shedding time in a sub-group of the infected children, because I believe that a short shedding time might explain why HMPV rarely has been detected in children without RTI. Lastly, I have examined HMPV and other respiratory viruses in day-care children.

The data was collected from three studies: A) The Childhood Airway Infections Research Group (CAIR) study (2006-2015); B) The HMPV shedding study (2012-2015) and C) The Day-care study (2012-2014). The Pediatric Emergency Department and wards, at the Department of Pediatrics, at St. Olav's Hospital, Trondheim University Hospital, were the main areas for studies A and B, and two day-care centers in Trondheim municipality for study C.

In the introduction, I will review the current knowledge of HMPV. In addition, I will also give a short overview of the most common respiratory viruses in children with RTI and in asymptomatic children.

Review of the current knowledge of HMPV

Since the discovery of HMPV, a large amount of scientific evidence has been published. I have tried to base this background section on the most important papers, the clinical studies with large sample sizes and those with a long duration. In addition, I have included data from all continents and even from adults to create a broad overview.

HMPV structure and replication

In 2001, HMPV was discovered by researchers from The Netherland's, and was named according to a genetic similarity with the avian metapneumovirus (AMPV) (1). Later analyses have suggested that HMPV originated from this avian virus approximately 200 years ago (16, 17). HMPV is an enveloped, negative-sense, single-stranded RNA virus in the *Pneumoviridae* family that includes RSV (1) (https://talk.ictvonline.org/taxonomy/). The genome size of HMPV ranges from 13.280 to 13.378 nucleotides, and contains at least 8 genes encoding nine proteins (17). The gene order in HMPV is 3'-N-P-M-F-M2 (M2-1/M2-2)-SH-G-L-5', with an average diameter of 200 nm (18, 19) (Fig 1). These viral proteins are nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix proteins (M2-1, M2-2), small hydrophobic protein (SH), attachment protein (G) and large RNA polymerase protein (L) (17, 19) (Fig 1). RSV is quite similar to HMPV, while RSV has two more genes (NS1 and NS2) and the order of the genes differs (17). The genomic organization of the HMPV genotypes A and B are identical, whereas differences in nucleotides define the genotypes (A and B) and subtypes (A1, A2, B1 and B2) (20-23).

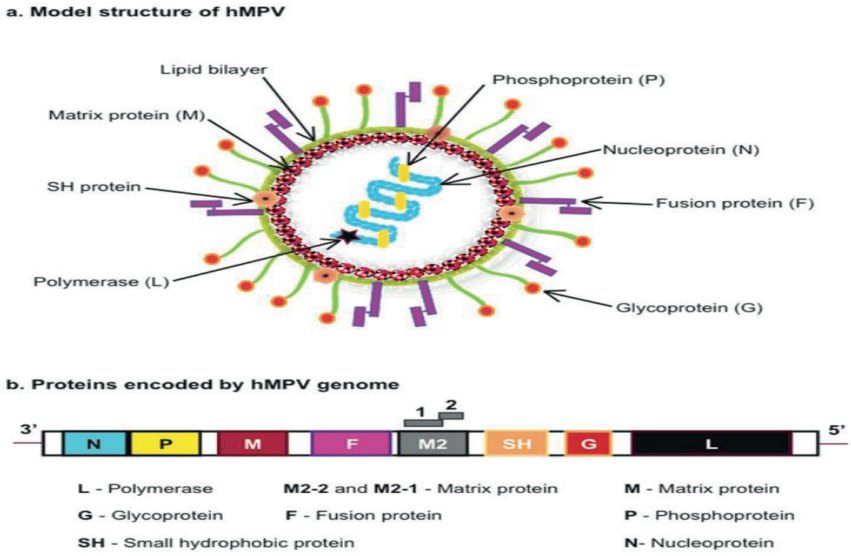

Fig 1. Structure and proteins encoded by human metapneumovirus (HMPV); (a) model structure of HMPV with viral proteins encoded by (b) the viral genome of HMPV.

HMPV attaches the host cell probably directed by the G protein, followed by fusion of the viral membrane with the host cell membrane mediated by the F protein (17). In addition, there is some evidence for the F protein to be involved in the attachment process (24). After the membrane fusion, the viral RNA genome is released into the cytoplasm and mRNAs are produced by transcription done by the viral L polymerase complex, which serves as a template for protein production (translation) by using the ribosomes of the host cell (17, 19). Full-length negative-sense, single-stranded RNAs (vRNA) are produced, in which N proteins have a major role for the packaging of linearized genomic RNA into helical complexes with M2-1 proteins and L proteins (18). The P-protein also has a central role in the assembly of the helical complex (18). The M2-2 protein is thought to play a role in shifting the balance of RNA synthesis from mRNA to vRNA (25), while the exact function of the SH protein has been difficult to find in experimental studies (17). Viral transcription, translation and replication occur in the cytoplasm of the host cell. It is therefore assumed that mature virions bud from the host cell, after assembly, along with a host-derived lipid bilayer envelope (17).

Pathophysiology and immune responses to HMPV

HMPV is thought to be transmitted by contact and droplets as with other RNA viruses (26), with an estimated incubation period of 7-9 days (27, 28). Moreover, some have analyzed the spread of HMPV within families, in which the time interval between symptom onset in a HMPV-positive index patient and the onset of symptoms in a contact patient was 5 days (29). HMPV primary targets are epithelial cells and leukocytes (18). Studies including humans and mice have shown that HMPV infection leads to damage in the respiratory tract, with necrosis of respiratory epithelial cells, along with a subsequent loss of ciliation, increased mucous production and inflammation (30-32). The epithelial cells, alveolar macrophages and dendritic cells are all important to sense HMPV and to mediate inflammation in mice (33). In humans, the innate immune response against respiratory infections are also dependent on these cells, together with neutrophils and lymphocytes (34). The epithelia cells serve as physical barrier, produce mucus that traps pathogens and remove it by movement of the ciliated epithelial cells. Once the pathogen has breached the epithelial layer, the early defense is mediated by the innate immune responses, and includes the production of cytokines, complement activation and phagocytic responses.

The detection of HMPV and other respiratory viruses in infected children is crucial in order to trigger the innate immune responses. The viruses may be detected extra- and intracellularly by several receptors (called pattern-recognition receptors) that recognize specific molecular structures (called pathogen-associated molecular patterns). Viral detection by these receptors triggers intracellular signaling pathways, thus leading to the production of cytokines, including type I interferons, which is essential for early antiviral defense (35). The immune responses thereafter are correlated to the child's age. In infants, both the innate and adaptive immune responses are immature. This involves a low production of tumor necrosis factor and type I interferons, immature dendritic cells, immature T cell function and poorly matured B cells (36). Furthermore, the infant immune responses may be skewed towards the T helper 2 phenotype, in which an early-life LRTI may lead to subsequent bronchial hyperreactivity and the development of asthma due to persistent T helper 2 activity (36). However, the impact of early viral infections on the development of asthma is complex, also including genetic and environmental factors (18, 36), and will not be further reviewed in detail.

Severe HMPV infections mostly affect children <2 years of age (11, 37). In healthy older children with a more mature immune system, HMPV and other respiratory virus

infections are mostly mild as upper RTI (URTI) and self-limited (38, 39). After an HMPV infection, the immune system produces antibodies for later protection. First, newborns receive maternally derived antibodies through the placenta during the last trimester as protection during early infancy. However, these antibodies decreases, with one study showing that HMPV antibodies were at the lowest level in infants between 3 and 5 months of age (40). After 13 months of age, the antibody levels gradually increased as evidence of exposure to HMPV (40). Furthermore, others have shown that the majority of children are HMPV seropositive at the age of 5 years, and approaches 100% by 5-10 years of age (1, 41, 42).

Laboratory diagnosis

Sample collection

HMPV and other respiratory viruses most often are diagnosed in respiratory specimens from nasopharynx, and then collected by swabs (NPS) or aspiration (NPA). Secretions from lower airways, such as sputum, tracheal aspirate and bronchoalveolar lavage, may also be used. However, the sampling procedures from lower airways may be uncomfortable or impossible to obtain without general anesthesia in children. Hence, respiratory samples from upper airways are mostly used for microbiological testing. Samples collected from the upper airways are shown to be well correlated with samples from lower airways for most respiratory viruses (43), and samples collected by swabs or by aspiration were reported with almost equal quality (44). Thus, viruses located in the lower airways are also mostly present in upper airways. Blood samples are primarily collected for measuring general markers of the host response to acute RTI, although serology may sometimes be used for microbiological diagnosis. Additionally, blood samples may be used to discover viremia by the use of polymerase chain reaction (PCR) tests.

Virus detection methods in respiratory specimens

HMPV was discovered in viral cultures using live cells from tertiary monkey kidney cells (1). Nonetheless, HMPV replicated slowly in these cells and very poorly in several other cell lines, so it could take 10-14 days before detection (1). In general, the viral culture detection methods are based on virus propagation in live cells: subsequently, the viruses have pathogenic effects on cells (cytopathogenic effect). The cytopathogenic effect patterns are related to specific virus types and identified by fluorescent antigen and visualized by light microscopy. Still, as previously mentioned, some disadvantages exist with this method, since some viruses do not grow in cell lines and others grow slowly. Hence, the sensitivity is low,

and it may take several days to obtain the results. HMPV is mostly cultured in tertiary monkey kidney cells, Vero cells, BEAS-2B cells, A549 cells, HepG2 cells and LLC-MK2 cells (kidney cells from the Macaca mulatta (monkey)) (17, 45), whereas the cytopathic effect pattern may not be easily visible.

The new molecular detection methods established in the 1990s, such as PCR tests, are based on the sequence-specific amplification of small gene material (RNA/DNA) from viruses and bacteria. PCR tests are the most sensitive methods for detecting HMPV and other respiratory pathogens (10), whereas PCR targeting the HMPV N gene is often used for an accurate detection of all HMPV subtypes (46). By PCR, several previously unknown pathogens such as human bocavirus (HBoV), new coronaviruses and human rhinovirus (HRV) C have been discovered (47). Prior to the PCR tests, the virus detection rate in children with RTI was low in less than 40%, while after its introduction is has increased to 72-95% (10). Several PCR methods exist, such as reverse transcriptase and real-time, in which the real-time PCR tests are mostly used. Moreover, singleplex (one target) or multiplex (several targets) PCR tests exist (10). Even so, due to the high sensitivity, some virus types and more than one virus (≥2, viral co-detection) are frequent in both asymptomatic (48, 49) and symptomatic children (48, 50). Previous studies in HMPV-infected children even found a high rate of such viral co-detections up to 53% (5, 11). Quantitative real-time PCR analyzes the viral loads and may be used to evaluate the timing of infection (i.e. high load in a new infection, low load in a previous infection), and has also been related to disease severity (10, 51). The enzyme linked immunosorbent assay (ELISA) and the immunofluorescence (IF) methods have been used for direct antigen staining of respiratory specimens for viral proteins, in which the ELISA method is mostly used. These detection methods are rapid and may produce results in hours. Even so, the sensitivity of such antigen tests are generally lower than PCR tests, and varies between viruses and commercial kits (52). On the other hand, some antigen tests for HMPV and RSV have exhibited a high sensitivity in 80-95%, with PCR tests as the gold standard (53-55).

Virus detection by serology

Serology testing, with the detection of an increased level of antiviral antibodies in paired serum samples (acute and convalescent), by ELISA or IF and measuring IgM and IgG levels, have been used to confirm a host response to recent HMPV infection (56), although the practical usefulness of serology in the diagnostics of an acute viral infection is limited (45, 52), and is mostly used in epidemiological studies (41).

HMPV epidemiology

HMPV has been detected worldwide, and the epidemiology of HMPV has been described in many studies. I have therefore made an overview of such studies presented in Tables 1, 2, 3 and Figure 2. In the Northern Hemisphere, most studies have reported HMPV outbreaks occurring mostly from December to May, while countries in the Southern Hemisphere may have activity all year or peaks from August to November (Tables 1 and 2). In some countries with a temperate climate, a biennial pattern has been observed, with alternating winter and spring seasons with a high HMPV activity (57, 58). Previous research has shown that HMPV genotypes A and B often co-circulate during outbreaks, whereas the dominant HMPV subtype may differ from one epidemic to the other (Table 3 and Fig 2). The HMPV subtype A1 has seldom been detected over the past 10 years, with the exception of several cases in Italy in 2009/10. The other subtypes (A2, A2a, A2b, B1 and B2) have been detected in all continents, with a varying distribution among countries and study years. The majority of studies have reported the circulation of 2-4 HMPV subtypes during the same season, whereas 1-2 subtypes were dominant, and these were displaced by others in every 1-3 years. HMPV has been detected in 1.7-21.9% of hospitalized patients with RTI or fever at all ages in studies covering long periods, with some variation from one season to another within the same area (Table 1). The majority of studies covering ≥2 respiratory seasons have detected HMPV in 4.8-11.2% of hospitalized children (Table 1). Even higher detection rates, up to 43%, have been shown during outbreaks among smaller groups of children (59). Quite similar HMPV detection rates, such as in hospitalized patients, have been reported in studies, including outpatients at all ages with RTI, fever or an influenza-like illness in 0.3-20.0% (Table 2). The majority of studies covering ≥2 respiratory seasons have detected HMPV in 4.4-5.2% of children with RTI treated as outpatients (Table 2). In summary, HMPV infections have been observed in all age groups, with the highest prevalence among children younger than 2 years of age (See "Peak age of HMPV-infected patients" in Table 1 and Table 2). In selected groups of young children with a mainly lower RTI (LRTI), either hospitalized (3, 11, 60) or treated as outpatients (12), the HMPV detection rates have been reported to be higher (8.6-20.0%) than in same-aged children with mostly URTI in the community (2.2-6.1%) (61, 62).

Table 1 Overview of studies reporting HMPV detection rate among hospitalized patients*

Age group in study	No. HMPV/total tested (%)	Seasonal HMPV variation (%)	Peak period of HMPV	Peak age HMPV-infected patients	Study design	Study period	Country (reference)
< 2 years	202/3576 (5.6)	NA†	Odd years: Des-Mar Even years: Mar-Jun	NA	Retrospective	Oct 1987 – Sep 2008	Austria (57) includes data from (63)
All ages < 3 years ≥ 3 years	143/4989 (2.9) 72/2579 (2.8) 71/2410 (2.9)	0.8-5.9 0.6-6.8 1.2-7.7	Feb-Mar in 4 seasons. Apr-May in 1 season.	< 1 year and > 60 years	Retrospective	Nov-May in 2001/02-2005/06	Sweden (64)
< 4 years	95/797 (11.9)	0-32.8	Feb-Mar	< 1 year	Retrospective	Oct-April in 2000/01-2009/10	Germany (65)
< 5 years	160/3320 (4.8)	2.9-8.8	Oct-Apr	< 1 year	Prospective	Jan 2007-Dec 2011	Kenya (4)
Children	168/1146 (14.7)	10.5-21.3	Sep-Nov	< 1 year	Prospective	2009-2011	Argentina (66)
< 2 years	109/1612 (6.8)	3.0-10.1	Odd years: Nov-Feb Even years: Mar-Jun	NA	Retrospective	Oct 2000-Oct 2007	Austria (63)
< 5 years	200/3490 (5.7)	4.0-9.0‡	Jan-Apr‡	< 2 years	Prospective	Nov-May in 2003/04-2008/09	US (2)
< 5 years	42/1104 (3.8)	3.3-4.2	Feb-May	< 2 years	Prospective	Oct 2001-Sep 2003	US (67)
< 3 years	66/440 (15.0)	8.7-20.8	Feb-Mar	NA	Prospective	Oct 2005-Sep 2007	US, Alaska (3)
< 5 years	48/347 (13.8)	4.7-25.3	Nov-Mar	< 2 years	Prospective	Oct-April in 2005/06-2006/07	Italy (68)
< 2 years	273/3168 (8.6)	NA	Feb-Apr	Median age 5.8 months	Prospective	Mar 2010-Mar 2013	Jordan (11)
< 3 years	96/2405 (4.0)	NA	Dec-May	< 12 months	Retrospective	Jul 2001-Jun 2005	Spain (69)

Table 1 Continued

Age group in study	No. HMPV/total tested (%)	Seasonal HMPV variation (%)	Peak period of HMPV	Peak age HMPV-infected patients	Study design	Study period	Country (reference)
≤ 14 years	45/661 (6.8)	NA	Dec-Jan	< 1 years	Prospective	Dec 2006-Nov 2008	China (70)
< 14 years	135/2613 (5.2)	NA	Winter-spring	< 1 year	Prospective	Sep 2007-Feb 2011	China (5)
< 5 years	68/516 (13.2)	NA	Nov-Mar	6-11 months	Prospective	Nov 2001-Oct 2002	Israel (71)
< 6 years	725/3313 (21.9)	NA	NA	Median age 13.0 months	Retrospective	Jan 2005-Apr 2010	Taiwan (72)
All ages	65/3858 (1.7)	0.2-4.3	May-Sep	≤ 5 years	Prospective	Jun 2007-Dec 2009	Cambodia (73)
Children	114/632 (18.0)	13.7-22.0	Dec-Jan	< 24 months	Retrospective	Oct-Apr in 2002/03-2003/04	Germany (74)
< 2 years	69/748 (9.2)	NA	Feb-Apr	Mean age 6.9 months	Prospective	Oct 2000-Jun 2003	Spain (75)
All ages	46/682 (6.7)	NA	Dec-Jan	< 2 years	Retrospective	Sep 2000-Feb 2002	The Netherland's (76)
All ages	596/6288 (9.5)	1.0-16.0	Apr-Oct	< 5 years	Prospective	Nov 2007-Dec 2012	Guatemala (77)
< 5 years	34/829 (4.1)	2.0-6.7	All year	< 1 year	Prospective	Jan 2010-Dec 2014	Bangladesh (78)
< 15 years	79/846 (9.3)¶ 79/2441 (3.2)‖	4.7-22.9¶	Nov-Apr	< 1 year	Retrospective	Oct-Apr in 2004/05-2009/10	Italy (79)
< 18 years	1975/38213 (5.2)	0.0-26.5**	Feb-Apr	< 2 years	Retrospective	Jul 2007-Jun 2013	US (80)
< 3 years	90/796 (11.3)	10.8-12.2	Dec-May	< 1 year	Prospective	Jul 2004-Jun 2007	Spain (60)
< 3 years	56/931 (6.0)	3.1-10.1	Dec-Mar	Median age 6 months	Prospective	Dec 2002-Apr 2004	France (81)
All ages	707/10025 (7.1)	NA	Aug-Oct	< 5 years	Prospective	Jan 2001-Dec 2004	Australia (7)

*Included patients with respiratory tract infection or fever. †Not available, however several studies showed the seasonal variation in relation to months in figures, but the exact seasonal variation was not possible to obtain. ‡Data was presented from the total group of both inpatients and outpatients. §Figures included both inpatients and outpatients. ¶Samples prior tested to be virus-negative. ‖All samples. **Monthly variation.

Table 2 Overview of studies with HMPV detection rates in patients* with respiratory tract infections

Age group in study	No. HMPV/total tested (%)	Seasonal HMPV variation (%)	Peak period of HMPV	Peak age HMPV-infected patients	Study design, area and inclusion	Study period	Country, reference
All ages	138/4549 (3.0)	1.2-6.8	Feb-Mar	< 4 years and > 60 years	Retrospective Outpatients with ILI†	Oct-Apr in 2000/01-2009/10	Germany (65)
All ages	5/1970 (0.3)	NA	Jun-Jul	<14 years	Prospective Outpatients with ILI†	Jan 2011-Dec 2013	China (82)
≤ 16 years	640/12299 (5.2)	1.1-10.0	Winter	Median age in inpatients 1.5 years, outpatients 3.6 years	Prospective Inpatients and outpatients	Jul-Jun in 2002/03-2005/06	Germany (83)
< 5 years	49/248 (20.0)	0-31	Dec-Apr	< 1 year	Prospective. Retrospective HMPV-testing of previous virus-negative samples. Outpatients with LRTI	1976-2001	US (12)
Children	198/3934 (5.0)	0.2-13.9	Winter in 2005/06 and 2007/08	Mean age in inpatients 2.6 years, outpatients 2.8 years	Prospective Inpatients and outpatients	Oct 2004-Apr 2008	Switzerland (84)
< 13 years	47/1338 (3.5)	NA	Jan-Mar	< 2 years	Prospective Outpatients (study clinic)	Oct 2000-May 2001	Finland (38)
< 3 years	127/892 (14.2)	NA	NA	6-11 months	Prospective Outpatients (home visits)	Mar 2009-Sep 2011	Peru (85)
≤ 15 years	28/525 (5.3)	NA	Feb-May	Median age of all included was 26 months	Prospective Outpatients	Jul 2011-May 2012	Mexico (86)
≤ 30 months	14/318 (4.4)	NA	Jan-May	Mean age 12 months	Prospective Outpatients (day care/ home visits)	Feb 2006-Apr 2008	US (87)
< 5 years	33/543 (6.1)	NA	Jun	Mean age 28.3 months	Prospective Outpatients‡	Jan 2003-Jan 2004	Australia (61)
< 3 years	87/3957 (2.2)	NA	Jun-Oct	6-11 months	Prospective Outpatients (home visits)	May 2009-Sep 2011	Peru (62)
< 15 years	171/3833 (4.4)	1.2-7.2	Dec-May	1-2 years	Prospective Outpatients (hospital clinic)	Jul 2008-Dec 2013	China (88)

*Patients included with respiratory tract infections or fever from differing study areas, as specified in the column "Study design, area and inclusion". Patients included from hospitals are denoted as inpatients. Patients included from hospital emergency departments, study clinics, private or public practices and community are denoted as outpatients. †Influenza-like-illness. ‡Excluded children with chronic diseases and those premature born.

Table 3 Overview of studies reporting HMPV genotypes and subtypes

Age group in study	Genotyped/tot. HMPV (%)	HMPV subtypes n (%)	Main findings	Study period	Country (reference)
< 2 years	189/202 (93.6)	A1 = 28 (15) A2a = 35 (19) A2b = 42 (22) B1 = 26 (14) B2 = 58 (31)	1-2 dominating subtype, displaced by another in every 1-3 years	Oct 1987- Sep 2008	Austria (57)
< 3 years	100/100 (100)	A1 = 7 (7), A2 = 53 (53) B1 = 12 (12) B2 = 28 (28)	2-3 subtypes co-circulated. A2 in all seasons. A1 in first two seasons. B1 in several years and peaked in 2004/05. B2 mainly in last 3 seasons.	Dec 2002- Mar 2009	France (89)
All ages	93/138 (67.4)	A1 = 12 (13) A2a = 13 (14) A2b = 36 (38) B1 = 13 (14) B2 = 21 (22)	2-4 subtypes co-circulated. A2a mainly in 2003/04. A2b in all seasons, except 2000/01 and 2006/07. A2b mainly in 2009/10. A1 in 2000/01-2002/2003, not detected later.	Oct-Apr in 2000/01-2009/10	Germany (65)
< 5 years	123/160 (76.9)	A2 = 91 (74), B1 = 4 (3) B2 = 28 (23)	2-3 subtypes co-circulated. A2 dominated all seasons.	Jan 2007- Dec 2011	Kenya (4)
< 15 years	145/171 (84.8)	A1 = 1 (1),A2b = 61 (42) B1 = 42 (29) B2 = 41 (28)	3 subtypes co-circulated each season. A2b dominated in 2008/09 and 2012/13.	July 2008- Dec 2013	China (88)
< 15 years	79/79 (100)	A1 = 14 (18) A2a = 21 (27) A2b = 19 (24) B1 = 14 (18) B2 = 11 (14)	1-5 subtypes co-circulated. A2 subtypes dominated in 2005/06 and 2006/07, B2 in 2007/08, B1 in 2008/09 and A1 in 2009/10.	Oct-April in 2004/05-2009/10	Italy (79)
< 3 years	151/163 (92.6)	A1 = 13 (9) A2a = 27 (18) A2b = 43 (28) B1 = 7 (5) B2 = 61 (40)	Multiple subtypes co-circulated in each season. Each of 5 subtypes was predominant during at least 1 season. A1 dominated in 2001-2003 (not detected after 2002/03), B2 in 2004/05, A2a and B2 in 2006/07, B2 in 2007/08, A2a in 2008/09 and A2b in 2009/10.	7 winter seasons 2001-2010	Canada (23)
All ages	640/727 (88.0)	A1 = 79 (12) A2 = 208 (32) B1 = 234 (37) B2 = 119 (19)	All subtypes co-circulated each year. A1 dominated in 2001 and A2 in 2002. B1 dominated in 2004.	Jan 2001- Dec 2004	Australia (90)
< 2 years	215/273 (78.8)	A2 = 117 (54.4) B1 = 11 (5.1) B2 = 87 (40.5)	Genotype A and B co-circulated. A2 dominated in 2010/11 and both A2 and B2 in 2011/12.	Mar 2010- Mar 2013	Jordan (11)

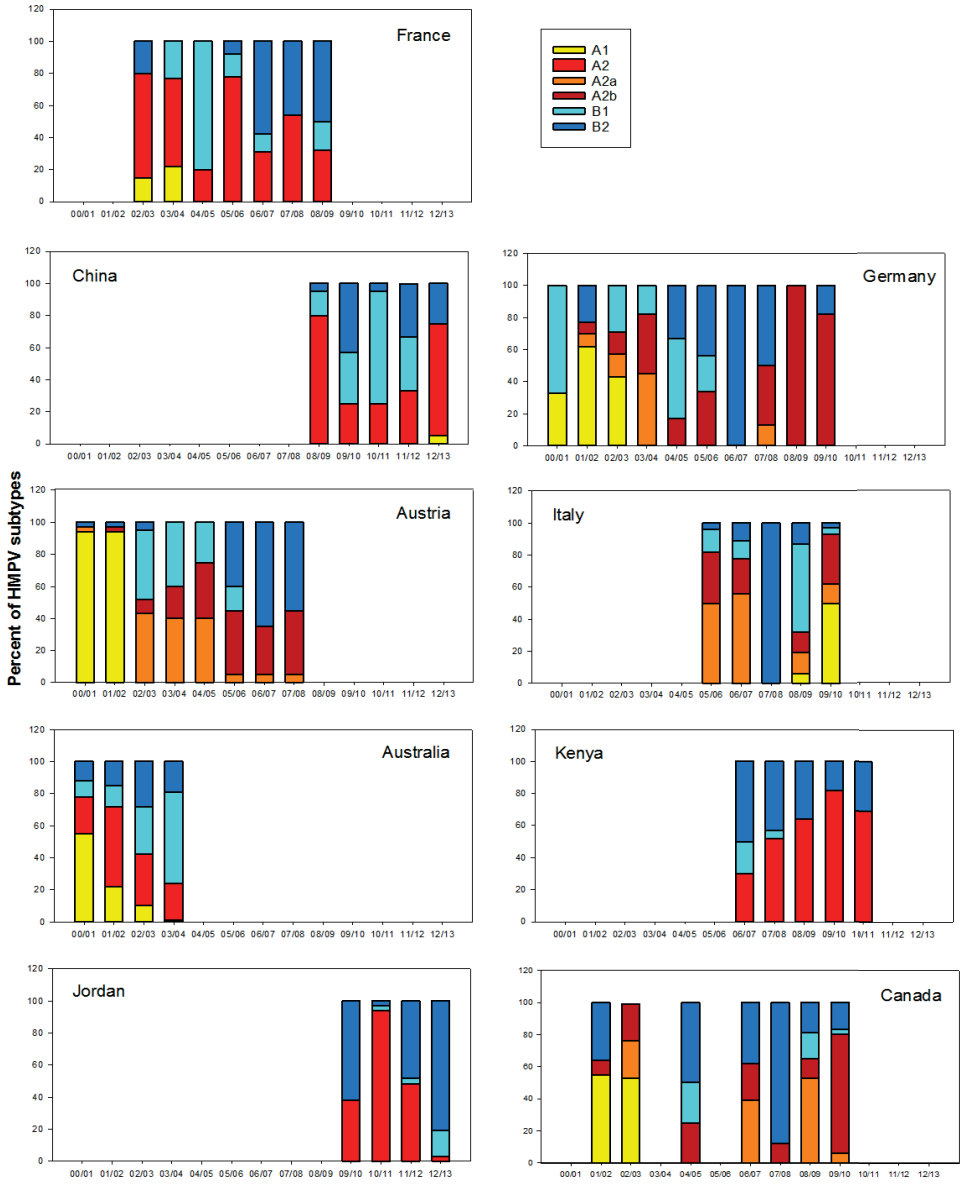

Fig 2. Distribution of HMPV subtypes from several countries according to season.

References related to these countries are shown in Table 3.

Incidence rates of HMPV infection

The studies reporting population-based incidence rates of HMPV-associated hospitalization are presented in Table 4. In three studies from the US, the average annual hospitalization rates were reported to be 1.0-1.2/1,000 children <5 years old, and higher rates were shown in the youngest children (2, 67, 80). A study from the UK (91) reported a slightly higher rate of 1.3/1,000 children <6 years old, while even higher rates were found in Spain (60) with 2.6/1,000 children <3 years old. In general, the rates were highest in the youngest children, <6 months and 6-11 months old, which was followed by a gradually decrease with an increasing age (Table 4). Thus, the reported hospitalization rates differed among countries and age, and only two studies were from Europe (60, 91). Additionally, some of the hospitalization rates were based on data from only 1 (91), 2 (67) or 3 (60) years. One study from Finland has reported incidence rates of HMPV infection among children in the community, treated as outpatients during 1 year in a study clinic, in which children <2 years of age had the highest rate in 76/1.000 children, with the rate decreasing with increasing age (38). A study with a longer duration from the US found that 55/1,000 children <5 years of age were treated as outpatients at a hospital with an HMPV infection (2). Consequently, the outpatient burden of HMPV infections has been higher than the hospitalization rates.

HMPV in asymptomatic children

Some studies have assessed HMPV among children without respiratory symptoms in the community, in which the majority showed a low detection rate of 0-1.3% (2, 6, 12, 48), while only one showed a higher rate in 7% (3). The criteria for being without respiratory symptoms (asymptomatic), have been based on information provided by caregivers, such as without cough, a stuffy nose, sore throat or fever during the last 1-2 weeks. Two studies have assessed HMPV in healthy children at the health-care station in relation to routine vaccination, in which symptoms were not exclusion criteria, and they also found a low HMPV detection rate of 0-5% (92, 93). One study also revealed that the HMPV viral loads in asymptomatic children were lower than in symptomatic cases (2). Among hospital controls at all ages, the HMPV detection rate was also low in 0.3% (76). Hence, in previous studies the HMPV detection rates among asymptomatic children have mostly been lower than in symptomatic patients. However, some of these studies had rather few children included (6, 12), one had a short duration (48) and only one measured HMPV viral loads (2).

Table 4 Overview of studies reporting incidence rates of HMPV-associated hospitalization

Age group in study	Average rate per 1000 per year, in age-groups	Inclusion criteria*	Study design and detection method	Study period	Country (reference)
< 5 years	< 6 months: 3 6-11 months: 2 12-23 months: 1 24-59 months: 0 0-59 months: 1	RTI or fever	Prospective PCR	2003-2009	US (2)
< 5 years	< 6 months: 4.9 6-11 months: 2.9 12-23 months: 0.7 24-59 months: 0.4 0-59 months: 1.2	RTI or fever	Prospective PCR	2001-2003	US (67)
< 18 years	< 6 months: 2 6-11 months: 2.5 12-23 months: 1.8 2-4 years: 0.47 0-59 months: 1.1 0-18 years: 0.36	Hospitalized ≥ 24 hours with RTI	Retrospective 2007-2009: antigen test. 2009-2013: PCR	2007-2013	US (80)
< 3 years	< 6 months: 6.7 6-11 months: 3.4 12-23 months: 1.8 24-35 months: 0.9 0-35 months: 2.6	RTI or fever	Prospective PCR	2004-2007	Spain (60)
< 6 years	0-11 months: 3.2 12-23 months: 2.4 24-47 months: 1.1 48-71 months: 0 0-71 months: 1.3	RTI, fever, acute febrile gastrointestinal illness, apnea or other life-threatening events	Prospective PCR	2001-2002	UK (91)
All ages	< 6 months: 2 6-23 months: 2 2-4 years: 0.3 < 5 years: 1.0	Fever or elevated white blood cell count according to age, and RTI symptoms from lower airways	Prospective PCR	2007-2012	Guatemala (77)
< 5 years	< 5 years: 0.4	RTI	Prospective PCR	2010-2014	Bangladesh (78)

*All studies had specified inclusion criterion as described, and all studies had exclusion of children treated with immune-suppression.

Shedding of HMPV

Some studies with a limited number of included children (range 7-16) have analyzed HMPV shedding time (94-97). The methods used in these analyses varied, and they reported somewhat differing results; median 5 days (94), 13 days (range 5-20) (97), 1-2 weeks (95), to more than 2-3 weeks (96). For this reason, the HMPV shedding time may be close to RSV and influenza virus (94, 98, 99), and shorter than some other respiratory viruses, i.e. HBoV (100), human adenovirus (HAdV) (101) and HRV (102), which occasionally may be detected from several weeks to months after recovery from a RTI. Nevertheless, the limited number of children and differing results from previous studies showed that the HMPV shedding time is somewhat unclarified.

Symptoms and clinical features of HMPV infection

HMPV is associated with several symptoms and diagnoses from the airways (Table 5), though the symptoms and clinical findings related to HMPV may be difficult to distinguish from several other respiratory viruses. The most common symptoms of HMPV infections are cough, fever, a stuffy nose, heavy breathing and a reduced appetite (Table 5), whereas symptoms of vomiting and diarrhea are more seldom (12, 37). Most children have symptoms of 2-6 days prior to medical evaluation, and diagnoses in upper airways such as rhinopharyngitis and otitis media are frequent, while acute laryngitis is rare (Table 5). HMPV-associated lower respiratory illnesses such as bronchiolitis, pneumonia and asthma exacerbations are also common, particularly among children in need of a hospital stay (Table 5).

In the general community, HMPV infections in healthy children and adults are mostly presented as URTI, influenza-like illness and more seldom LRTI, with symptoms reported to last for 8-13 days (13, 38, 39, 61). In a prospective, 1-year follow-up study from the community in Finland, 10% of HMPV-positive children developed symptoms of LRTI (38). In a prospective birth cohort of 217 healthy children in Denmark, none of the HMPV-infected children <1 year old were hospitalized and only a few developed symptoms from lower airways (103).

Table 5 Overview of studies reporting symptoms and diagnoses in children with HMPV infections

Data	Reference												
	(38)	(72)	(37)	(12)	(67)	(104)	(105)	(106)	(74)	(107)	(81)	(9)	(108)
No. HMPV cases	39*	113*	62†	49†	29*	238†	61†	91*	48*	144†	51*	50†	54*
Study site	Study clinic	Hospital	Hospital	Outpatients‡	Hospital	Hospital	Hospital	Hospital	Hospital	Hospital	Hospital	Hospital	Outpatients
Symptom:													
cough	97%	98%	100%	90%	86%	92%	**	96%	65%	99%	67%	90%	98%
fever	72%	84%	92%	52%	76%	84%	90%	60%	38%	42%	39%	86%	100%
heavy breathing	**	**	84%	**	93%	59%	**	55%	65%	**	**	80%	40%
stuffed nose	90%	73%	76%	88%	72%	81%	90%	74%	**	**	67%	44%	**
reduced appetite	**	**	85%	33%	72%	47%	51%	**	**	**	39%	48%	**
wheezing	10%	57%	89%	22%	38%	56%	**	55%	**	77%	**	56%	**
ear pain	**	**	19%	**	**	17%	**	**	**	**	**	**	**
apnea	**	**	15%	**	**	**	**	9%	15%	**	**	4%	**
Symptoms-days	**	**	2.0 (median)	4.4 (mean)	**	**	5.8 (mean)	**	**	**	**	**	**
Upper RTI§:		1%¶			3%¶		**	**	20.8%	3%		16%‖ 67%‖‖	57%
rhinopharyngitis	90%	**	2%	**	**	54%	**	**	**	**	29%	12%	
tonsillitis	**	**	**	**	**	**	**	**	**	**	**	**	2%
acute laryngitis	8%	**	2%	18%	**	**	10%	**	8%	**	**	4%	0%
otitis media	41%	**	48%	37%	**	19%	**	**	8%	**	16%	**	
Lower RTI:								100%					
bronchiolitis	**	77%	56%	59%	38%	48%	7%	**	48%	19%	51%	48%	20%††
pneumonia	3%	22%	29%	8%	24%	15%	43%	**	10%	61%	**	34%	4%
asthma exacerbation	**	**	16%	14%	24%	15%	39%	**	6%	16%	12%	**	
other	**	**	**	**	10%	**	1%	**	**	**	8%	**	20%

*Data presented from single HMPV infections. †HMPV infections included both single virus and co-detections of other viruses. ‡Outpatients with lower respiratory tract infections. **Not reported. §Respiratory tract infection. ¶Isolated URTI diagnosis. ‖Isolated URTI diagnoses as specified below. ‖‖67% of children with LRTI had URTI, without specifying the diagnoses in upper airways. ††Bronchiolitis and asthma exacerbation.
Percent of diagnoses may be >100% since several studies allowed more than one diagnosis.

Disease severity of HMPV infection

To evaluate disease severity and risk factors for severe HMPV infection, a comparison of baseline characteristics, presence of comorbidities and clinical manifestations related to selected outcome variables have been performed. First, some studies have compared outpatients vs. hospitalized cases (2, 37). Clinical findings of isolated URTI and influenza-like illness in otherwise healthy older children seldom needed hospitalization, while LRTI in young children more often needed a hospital stay (2, 37). Secondly, among hospitalized children with HMPV infections, somewhat similar comparisons have been made (2, 11, 37, 104, 106, 109, 110). This often included outcome variables such as a need of an oxygen supply, respiratory support, admission to a Pediatric intensive care unit (PICU), duration of hospital stay and mortality (11, 37, 106, 110-112). Variables such as HMPV genotypes (5, 11, 37, 69, 70, 72, 89) and viral co-detections (5, 11, 111, 112) have also been included. The criteria used to classify different LRTI diagnoses, including clinical manifestations and supplemental investigations (as radiogram), differed in various studies (11, 37, 111, 112), making a direct comparison difficult. Overall, I concluded that several risk factors for severe HMPV infection among hospitalized children have been defined in studies using various disease severity measures (outcome).

Risk factors for severe HMPV infection

Several studies have shown that the population at risk for severe HMPV infection is young children and those <2 years of age in particular (Table 1), but some have shown that children <6 months old developed a more severe disease than older children (11, 37). Others have reported that slightly older infants aged 6-12 months were also at high risk (104). Some studies found that children with a history of prematurity and chronic diseases were also at high risk for severe disease (2, 37, 104, 106, 109, 110). It has also been shown, that chronic diseases such as asthma, bronchopulmonary dysplasia, neuromuscular diseases, congenital heart diseases, trisomy 21 and other chronic lung diseases have each been related to an increased disease severity (2, 37, 104, 106, 109, 110). Some have also demonstrated an increased risk among female gender (37, 110), while this has not been confirmed by others (11, 104). Individuals with impaired immunity at all ages, especially those treated with immunosuppressive drugs, are at a high risk for LRTI, with a high mortality rate due to HMPV infection (113, 114). Similar consequences of HMPV infection have also been shown in elderly (115) and adults with chronic diseases (13).

Clinical manifestations related to HMPV genotypes

Several studies have evaluated the clinical findings and disease severity related to HMPV genotypes A and B among children, with most hospital-based studies revealing quite similar manifestations and disease severity in different HMPV genotypes (5, 70, 72, 89). In addition, when including patients older than 18 years, no differences between genotypes were shown (116). However, a few studies restricted to children <3 years of age, and using different outcomes, have shown that either genotype A (11, 69) or genotype B (37) may cause more severe disease in hospitalized children. Hence, previous research has shown rather diverging results in relation to HMPV genotypes and disease severity.

Clinical manifestations related to viral co-detections

By using sensitive PCR tests, viral co-detections in hospitalized children with HMPV infection are common (5, 11). Some studies in HMPV-infected children found that such viral co-detections were associated with an increased disease severity in some selected groups of children during limited periods (111, 112), but this was not confirmed in others with a broader inclusion (5, 11). When including several viral detections methods such as virus culture, immunofluorescence and PCR, there were no differences in disease severity between HMPV detected as a single virus and HMPV with other viruses co-detected (71, 117). In total, previous research has shown different results in terms of whether viral co-detections in HMPV-infected children were related to clinical manifestations and disease severity.

Disease severity related to HMPV viral load

This theme has only previously been studied in a few studies. The HMPV viral loads among children ≤3 years old with RTI were found to be higher among inpatients (hospitalized) than outpatients, but high viral loads were not associated with a more severe disease among those hospitalized (118). Another study found a somewhat similar result (119). In a third study, high HMPV viral loads were associated with a hospital stay >2 days, but not with PICU admission or oxygen supply (120). Others found that LRTI was associated with higher HMPV viral loads than URTI (121). In summary, it is most unclear at the moment whether HMPV viral load is associated with disease severity, although a few studies have suggested that there may be an association.

Recurrent HMPV infections

As previously mentioned, the majority of children are HMPV-seropositive already as early as the age of 5 years. However, the protective effect of these antibodies may vary, even though a

high seroprevalence at all ages after the age of 5 (122). Data from experimental studies have suggested that certain HMPV subtypes may not stimulate an adequate immune response in all cell types (123). Hence, recurrent HMPV infections may occur due to different HMPV subtypes (12, 124, 125). In addition, some researchers have suggested that the second HMPV infection was more likely to be limited to the upper airways than the lower (12, 125). Furthermore, one study reported episodes of recurrent URTI with homologous or heterologous HMPV subtypes within weeks or months, suggesting that immune responses after URTI were limited and transient (125). Regardless of HMPV subtypes, in a retrospective hospital-based study covering 6 years of children with RTI, recurrent HMPV infections were quite seldom in 3% (80). Outside a hospital setting, others have shown that HMPV may cause mild RTI in both otherwise healthy children >5 years old (38) and in adults (13). Moreover, studies in children (113) and adults (114) with impaired immunity have revealed episodes of severe HMPV infections. Thus, previous studies have shown that recurrent HMPV infections may occur. Yet, a limited amount of data of recurrent HMPV infections in need of a hospital stay in otherwise healthy children exists.

HMPV and asthma

HMPV has the ability to exacerbate asthma in both children (67, 105, 126) and adults (127), and HMPV has also been detected in young children hospitalized with wheezing (128). A Spanish study revealed that hospitalization due to HMPV-bronchiolitis during the first two years of life was an important risk factor for asthma at the age of 5 (129). Nonetheless, the data from the Spanish study was based on physician-reported symptoms of asthma and wheezing, and without lung-function tests (129). In addition, only 23 HMPV-infected children were included, and nearly 40% were prematurely born (129).

Several studies have examined the association between HRV and RSV infections in early life and the development of asthma (130-132), whereas similar information for HMPV has been limited so far. Further studies of viral RTI and asthma, including genetics and immunology, are needed (133).

Phylogenetic analysis of HMPV

Many viruses, in specific RNA viruses may rapidly change their nucleotides because of high mutation rates (17, 134). Hence, new genetic pattern in viruses may develop and therefore escape from human immunity, and this may be an additional challenge related to the

development vaccines and anti-viral treatment (23). As previously stated, HMPV originated from AMPV approximately 200 years ago, while the current HMPV appeared about 100 years ago (17). Each of the two main genotypes (A and B) seems to have occurred during the last 30-50 years, whereas each of the subtypes is probably less than 30 years old (17). It is of importance to delineate which virus strains that circulate in various parts of the world, and phylogenetic analyses of the isolated HMPV samples in our cohort was therefore performed.

HMPV treatment and prevention

Viral RTI, including HMPV infections, are mostly URTI and self-limited in healthy children, and the majority may be cared for at home. Unfortunately, no specific anti-viral treatment is available. In many cases, anti-pyretic and nose drops (decongestant or NaCl) are helpful. Moreover, antibiotics may be given as treatment for otitis media in young children (38).

For children with severe HMPV infections, other treatment options are available at hospitals, with supporting treatment such as inhalations and oxygen are often provided to the majority (37, 105, 118). In addition, some studies reported that half or more of the hospitalized children received antibiotics (37, 105, 118). Invasive and non-invasive respiratory support are treatment options for those with the most severe diseases, and often at the PICU for 3.2-11.5% of HMPV-infected hospitalized children (37, 105). Antiviral treatment is also mostly never or seldom provided (37, 105, 118). Additionally, the antiviral nucleoside ribavirin has been tested in vitro and in animals with activity against HMPV (135). However, due to considerable side effects, this treatment has mostly been offered to severely affected transplant patients (135), and even to a young girl with acute lymphoblastic leukemia and immune suppression (136). The young girl was successfully treated.

Some therapeutics and vaccines have shown promising results in animal models (18, 135, 137), including immunoglobulins (138) and HMPV viral protein-based subunit vaccines (139). Thus, no vaccine or specific treatment against HMPV exists.

Other respiratory viruses in children with RTI

RTI are common in childhood, with an average of 5.0-6.2 RTI episodes per child-year in children <5 years old previously shown (61, 62). A recent study followed 154 children, and found an average of 13 RTI episodes and almost 5 months with respiratory symptoms per child during the first 2- years of life (140). In a birth cohort, nearly one-third of the RTI episodes during the first year of life were LRTI (141). Moreover, in a large prospective study

of 2009 healthy children followed to <5 years of age during 1976-2001, 23% developed one or more episodes of LRTI (12). Hence, the majority of RTI episodes may cause a mild disease such as URTI, while fewer may develop a severe disease like LRTI. Nonetheless, the worldwide burden of severe RTI and deaths due to RTI has revealed high figures (142).

Several respiratory viruses may cause RTI in children with quite similar symptoms and clinical findings. In this short overview, I will therefore not go into such details for each virus, but instead present an overview of viral detection rates in children's RTI at hospitals and in the community. Hereafter, I will present more information on the *Picornaviridae* family and RSV.

From hospital-based studies, the following viral detection rates have been found: RSV in 19-72%, HRV in 9-44%, HAdV in 6-30%, parainfluenza viruses (PIV) in 2-24%, influenza viruses in 1-17%, HBoV in 8-13%, human corona viruses (HCoV) in 1-7% and human enterovirus (HEV) in 2-5% (3, 5, 50, 92, 143). Overall, 62-94% of children with RTI had one or more viruses in these studies (3, 5, 50, 92, 143). In the community, the following viral rates have been shown in children <5 years of age with RTI: HRV in 18-71%, HBoV in 0.4-14%, HAdV in 8-22%, HEV in 9-10%, RSV in 2-11%, PIV in 4-12%, influenza viruses in 2-7% and HCoV in 1-15% (61, 87, 144, 145). In total, 60-92% had one or more viruses (61, 87, 144, 145).

The figures varied among studies, and the distribution of respiratory viruses may differ from children with RTI in the community to hospitalized cases. Moreover, the hospitalized cases may often have a more severe disease than those in the community. In general, HRV is a common virus in both mild and severe RTI, while RSV is more often detected in severe cases. In addition, influenza viruses and PIV may be more frequent in hospitalized children than in those with milder RTI. Among hospitalized children, RSV and HRV have mostly been detected more frequently than HMPV (4.8-11.2%), whereas the HMPV detection rate has often been quite similar to PIV and influenza viruses.

Picornavirdae

The large *Picornaviridae* family is comprised of HRV, HEV and human parechovirus (HPeV), including more than 100 HRV serotypes (146). HRV is often detected in common cold (144), and one study showed that 74 distinct HRV types circulated in the community within one year, thereby causing recurrent RTI in preschool-aged children with different HRV types (147). As previously mentioned, HRV is a common respiratory pathogen that may cause

both mild and severe infections. The several enteroviruses may cause a variety of manifestations such as RTI, gastroenteritis, meningitis, neonatal systemic illness, rash, paralysis, myopericarditis and hand, foot and mouth disease (148). Most of the HPeV types have been discovered recently, and their clinical relevance in RTI is not yet established. HPeV in children has previously only been examined in a few studies, with low detection rates from 1.6% to 2.1% in children hospitalized with RTI (149, 150). Serological studies have documented that most Finnish children may be infected with HPeV1 (83%) and HPeV2 (91%) before the age of 5 years (151), while HPeV3 has been strongly related to sepsis-like disease and encephalitis, but not RTI, in infants (152). Several picornaviruses have even been detected in asymptomatic children (see later section).

Respiratory syncytial virus

Among many infectious pathogens, RSV has been associated with severe RTI, and has been detected in 60-70% of hospitalized young children with bronchiolitis (51, 153). Globally, the burden of RSV is enormous, causing an estimated 66,000-199,000 deaths among children <5 years of age in 2005, and >3 million children in this age group were hospitalized (154). Furthermore, the reported incidence rates of hospitalization of RSV-associated disease from several countries varied from 3.2- to 42.7/1,000 children in children <1 year (155). Going into more detail, this rate was 21.7/1,000 children in Norway (156), 41.4/1,000 children in Spain (157) and 26.0/1,000 children in the US (158). The outpatient burden of RSV infection in children <3 years of age was 275/1,000 children in Finland (159). Studies from the Northern Hemisphere have shown that RSV mostly circulates in winter and early spring (63, 83, 84). Two main RSV genotypes (A and B) exist, and they may co-circulate during epidemics (160, 161), in which quite similar clinical manifestations in RSV A and B (160).

It is characteristic with RSV that the majority of RSV-infected hospitalized children are previously healthy (162). In addition, some risk factors for severe RSV infection have been shown, such as a young age, prematurity, chronic lung disease, chronic heart disease and severe neurological disabilities (37, 106, 162-165). High RSV viral loads have been associated with more severe disease among young children hospitalized with bronchiolitis (51), though this was not confirmed in others when older hospitalized children were included (119). A previous Norwegian study showed that hospitalization with RSV-bronchiolitis during the first year of life was associated with bronchial hyper-reactivity at the age of 11 years (132). However, the RSV-negative bronchiolitis group in the same study had similar

findings and an increased risk for asthma (132). Hence, as stated earlier, the development of asthma is complex. As for HMPV, no specific treatment for RSV infection exists, while ribavirin has been offered in some severe cases (166). By contrast, new antiviral treatment has entered clinical trials (167). The prophylactic use of palivizumab (a specific RSV immunoglobulin) in selected high-risk children has also been used for nearly two decades (168), and reduced the frequency of severe RSV disease in this group (169). A lot of research has been done in order to develop RSV vaccines, in which recent papers have highlighted the possibility of future vaccines for use during pregnancy (170, 171).

Comparing HMPV and RSV infections

Among hospitalized children, RSV has been detected more often than HMPV (3, 5, 11, 37): consequently, the reported hospitalization rates were higher due to RSV infection (156-158) than HMPV (2, 60, 80). Children hospitalized with RSV infection are often younger, and more seldom have comorbidities than those with HMPV (2, 37, 71, 104, 105). Most studies have reported quite similar clinical manifestations and disease severity in HMPV- and RSV-infected children (71, 74, 172), whereas some have reported a more severe disease in those with RSV (37, 173). Risk factors for severe HMPV and RSV infections are described earlier in their respective sections. Moreover, most studies have separately dealt with risk factors for severe HMPV (2, 11, 104, 109, 110) and RSV infections (162-165), and only a few have compared them directly (37, 106). In addition, the definition of severe disease differed among studies by the use of differing outcome measures (11, 37, 106, 110, 163, 164). As a result, a comparison of risk factors for severe RTI in HMPV- and RSV-infected children in the same study using the same disease severity criteria for both viruses is needed.

Some early studies have reported that children who tested positive for both viruses might have a more severe disease than those with only one virus (111, 112). However, this has not been confirmed later (5, 11).

Respiratory viruses in asymptomatic children

By using PCR tests, several respiratory viruses have been detected in asymptomatic children in the community (3, 48, 87, 174), with two studies recruiting normal healthy children during a childhood immunization program with a planned vaccination (92, 93). The following viral detection rates were found in <7 years old children: HRV in 21-44%, HBoV in 4.3-21%,

HAdV in 0-20%, HCoV in 4-12%, HEV in 3-15%, HPeV in 9%, PIV in 0.5-6%, influenza viruses in 0-4% and RSV in 0-6% (3, 48, 87, 92, 93, 174). Overall, 35-67% of these children had one or more viruses. The frequent detection of several viruses by PCR in these children has been discussed, as to whether prolonged shedding, asymptomatic infection or latent infection may be a cause.

Viral co-detection

Since simultaneous molecular diagnostics for 10 viruses or more are available in clinical settings, recent papers have reported a high rate of detecting more than one virus in children with RTI (11, 175) and healthy controls (92). The rates of viral co-detections were higher among cases than controls in some studies (3, 48, 92). Others reported that some virus combinations may appear more frequently than others in both children with and without RTI (93, 175-177), and during the progress of a RTI more viruses may appear (175). In particular, HRV, HAdV, HEV, HPeV and HBoV may often be involved in viral co-detections (93, 175).

The clinical impact of viral co-detection in children's RTI has been discussed, with some studies finding a more severe disease (178), some finding no difference (117, 179) and even one study finding less severe disease with viral co-detections (180). Moreover, the rate of viral co-detection may be related to age, with higher rates in children aged 13-24 months old, than those aged 8-12 months or 25-36 months old (181). Due to frequent viral findings in asymptomatic/healthy and symptomatic children, some studies have compared the groups in statistical analyses, adjusting for age and viral findings (single-virus detection and viral co-detection), with severe RTI (cases) vs. no RTI (controls) as the outcome. Their analyses showed a high probability of severe RTI for influenza viruses, PIV, RSV and HMPV (48, 92).

2 Aims of the study

Principal objective

The main aim of the project was to study clinical manifestations and virological features associated with HMPV in children with and without respiratory tract infections.

Specific objectives

- To assess the burden of HMPV respiratory tract infections in hospitalized children compared to RSV: Paper I.
- To study the HMPV shedding time in children with acute HMPV respiratory tract infections and the occurrence of HMPV in asymptomatic children, to get an impression of HMPV in non-infected children: Paper I.
- To study if clinical manifestations in hospitalized children with HMPV lower respiratory tract infections were related to viral co-detections and HMPV genotypes: Paper II.
- To study if clinical manifestations and risk factors differed in HMPV- and RSV-infected hospitalized children with lower respiratory tract infections: Paper II.
- To study the occurrence of HMPV and other respiratory viruses in apparently healthy children attending day care: Paper III.

3 Materials and Methods

Three studies were performed: A) The CAIR study (Papers I and II); B) The HMPV shedding study (Paper I); and C) The Day-care study (Paper III).

The majority of the included children was sampled with respiratory secretions, and underwent clinical examinations. In addition, baseline characteristics, plus past and current medical history were registered. A detailed overview of each of the three paper included in the thesis is presented in Table 6, including the paper's topic, area, design, study period, inclusion criteria, type of respiratory samples, number of included children and analyses.

Study area and design

The three studies were performed in the municipality of Trondheim, Sør-Trøndelag County, Norway. The data included in the CAIR study was prospectively collected from children admitted with acute RTI to the Pediatric Emergency Department and the wards at the Department of Pediatrics and from healthy controls admitted to same-day surgery at the Department of Pediatrics, St. Olav's Hospital, Trondheim University Hospital, within the period from November 2006 to July 2015. These different locations in the Department of Pediatrics will further be named as the Department of Pediatrics (DP).

The data for the HMPV shedding analysis was collected from some of the children hospitalized with acute RTI and HMPV at the DP, St. Olav's Hospital and during home- visits from April 2012 to July 2015. Each of the included children was sampled with respiratory specimens at admittance and follow-up respiratory samples during the hospitalization period, and regularly after discharge. The information on the children in the HMPV shedding study is presented under the heading "Department of Pediatrics, St. Olav's Hospital" in the Materials and Methods section.

Data in the day-care study was collected from a cohort of children attending two day-care centers located in Trondheim, during 4 visits, in March 2012, October 2012, November 2013 and February 2014. The study was performed during the day in the day-care area.

St. Olav's Hospital is a tertiary hospital for the region of mid-Norway, and the only hospital with a pediatric department in Sør-Trøndelag County. The hospital provides care for 58.443 children younger than 16 years and 18.768 children younger than 5 years of age (Statistics Norway).

Table 6. Overview for each of the three papers included in the thesis.

Paper	Topic	Area	Study	Design	Study period	Inclusion criteria	Respiratory samples	Microbiological analyses	Statistical analyses	Included (n)
I	Epidemiology: Viruses in RTI	PD[a]	CAIR study	Prospective	Nov. 06-Jul. 15	Admitted[b] with RTI	NPA	Viral culture PCR	Descriptive[c]	3650
	Epidemiology: HMPV genotypes in RTI	PD	CAIR study	Prospective	Nov. 06-Jul. 15	Admitted with RTI	NPA	Viral culture PCR HMPV-genotyping	Descriptive	222
	Hospitalization rates of LRTI	PD	CAIR study	Prospective	Aug. 07-Jul. 14	Hospitalized[d] with LRTI: HMPV, RSV	NPA	Viral culture PCR	Descriptive	900
	HMPV shedding during RTI	PD and home-visits	HMPV shedding study	Prospective, longitudinal	Apr. 12-Jul. 15	Hospitalized with RTI and HMPV	NPA, NPS	Viral culture PCR	Descriptive Kaplan-Meier analysis	32
	Viruses in healthy controls	PD	CAIR study	Prospective	Jun. 07-Apr. 15	Healthy hospital controls	NPA	Viral culture PCR	Descriptive	339
II	Clinical manifestations in LRTI	PD	CAIR study	Prospective	Nov. 06-Jul. 15	Hospitalized with LRTI: HMPV, RSV	NPA	Viral culture PCR	Descriptive Logistic regression	1041
	Clinical manifestations in HMPV genotypes	PD	CAIR study	Prospective	Nov. 06-Jul. 15	Hospitalized with LRTI and HMPV	NPA	Viral culture PCR HMPV-genotyping	Descriptive	147
III	Viruses and clinical manifestation in day-care children	Two day-care centers, Trondheim	Day-care study	Prospective, cross-sectional, cohort	Mar. 12, Oct. 12, Nov. 13, Feb. 14	Children in day care	NPS	PCR	Descriptive Monte Carlo simul. test GLMM	161 children 368 inclusions

[a]Department of Pediatrics (Pediatric Emergency Department at the Department of Pediatrics and wards at the Department of Pediatrics), St. Olav's Hospital. [b]Amitted to hospital, included both children treated as outpatients (< 24 hours hospital stay) and those hospitalized (≥ 24 hours hospital stay). [c]Descriptive statistics included also comparison of means and medians, and comparison of categorical-, ordinal- and continuous variables. [d]≥ 24 hours hospital stay. RTI indicates respiratory tract infections; PCR, polymerase chain reaction; NPA, nasopharyngeal aspirates; HMPV, human metapneumovirus; LRTI, lower respiratory tract infections; RSV, respiratory syncytial virus; NPS, nasopharyngeal samples; GLMM, generalized linear mixed-effect models.

Childhood Airway Infections Research Group

CAIR was established in collaboration between the Department of Pediatrics and Department of Medical Microbiology at St. Olav's Hospital, and the Department of Laboratory Medicine, Children's and Women's Health, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim. The aim of the CAIR collaboration was research on respiratory viruses in childhood. The CAIR project started in November 2006 and is still ongoing. A part of the data from the CAIR project is included in the thesis, and is named the CAIR study. Children admitted to the DP for acute RTI and healthy hospital controls were enrolled in the study, and a database and biobank were established for the storage of information and respiratory specimens. However, there has been some delay in entering data to the database, so therefore the main study cohorts of included children differed in Papers I and II from the same study periods.

Study populations

Papers I and II: Department of Pediatrics, St. Olav's Hospital

Children aged <16 years admitted with acute RTI were enrolled in the study from November 2006 to July 2015. Children with cytostatic and immune-suppressive treatment and children hospitalized primarily due to diseases other than acute RTI were excluded. Some children with distinct RTI episodes were included more than one time, whereas recurrent hospitalizations due to the same RTI were only registered once.

In Paper I, a total of 3,650 children (main study cohort) were enrolled out of 4,111 children admitted with acute RTI.

In Paper II, a total of 3,214 children (main study cohort) were enrolled out of 3,932 children admitted with acute RTI. From the main study cohort, we selectively included HMPV- and RSV-infected children with LRTI, and with a hospital stay of ≥24 hours. Consequently, outpatients (<24-hour hospital stay) (n = 1,014), children hospitalized with URTI (n = 384) and children hospitalized (≥24-hour hospital stay) with LRTI and viruses other than HMPV or RSV and virus-negatives (n = 775), were excluded. Hence, a total of 1,041 HMPV- and RSV-infected children with LRTI were included in Paper II.

Some of the children hospitalized for 24 hours or more with an acute HMPV infection (n = 32) were enrolled for analysis of the HMPV shedding time (Paper I).

From June 2007 to April 2015, children aged <16 years hospitalized for elective surgery were enrolled as healthy controls. Children in the control group should be afebrile and without airways symptoms due to the surgery in general anesthesia. None of them were admitted for ear, nose and throat surgery. Several (n = 305) of the included controls reported respiratory symptoms during the last 2 weeks, or at inclusion, and were therefore excluded for the purpose of the study. In all, 339 remaining asymptomatic controls were included (Paper I).

Written, informed consent to participate were collected from caregivers and from children ≥12 years from most of the children during their hospital stay, but some children with acute RTI were enrolled after hospital discharge after passive consent.

Paper III: Day-care centers

Children aged 1-6.3 years old were enrolled from two day-care centers. The number of children varied from 110 to 132 at each visit, and the children were organized into 5 or 6 sections with the youngest children and 4 sections for the oldest children. Each child could be included only once during each visit and the exclusion criterion was previous nasal bleeding. In total, 161 children participated in the study one or more times (median 2, range 1-4), which resulted in 368 out of 484 possible inclusions (76.0%) (Fig 1 in Paper III).

The sections with the oldest children were visited 1 week before each visit, in which a clinical examination and sampling of respiratory secretion was demonstrated on a large doll in order to prepare the children and to achieve confidence (Fig 3). After the demonstration, the children received permission to examine the doll and each other with the medical equipment. Informed, written content from caregivers was collected on behalf of the children for each study visit.

Fig 3. The doll named Laura, used for the demonstration of clinical examination and the sampling of respiratory secretion for children in day care.

Baseline characteristics, clinical examination and diagnostic criteria

Papers I and II: Department of Pediatrics, St. Olav's Hospital

We collected baseline characteristics and past and current medical history from a questionnaire filled out by caregivers. In addition, some children with acute RTI were enrolled after hospital discharge, in which the information was abstracted from the hospital medical records. Caregivers of children enrolled for HMPV shedding analysis filled out an additional questionnaire about recent RTI symptoms at each visit.

Children admitted with RTI were routinely examined at the discretion of medical doctors at the PD, and clinical information was abstracted from the hospital medical records for the purpose of the study. Clinical findings and diagnoses were subsequently reviewed for the need for detailed categories. These categories were URTI, LRTI or combined URTI and LRTI. URTI included one or more clinical manifestations such as tonsillitis, otitis media, rhino-pharyngitis and acute laryngitis. LRTI was categorized into five diagnoses based on clinical manifestations and radiological findings, such as bronchiolitis (children <2 years old), obstructive bronchitis (children ≥2 years old), pneumonia, asthma exacerbation and unspecified LRTI. Combined URTI and LRTI had clinical manifestations in both upper and lower airways, and for details of LRTI diagnoses, see Paper II.

Children included in the HMPV shedding analysis were also clinically examined at each visit in order to evaluate changes in clinical manifestations during the HMPV infection.

Children in the control group were not clinically examined for the purpose of the study, but their caregivers confirmed the absence of respiratory symptoms.

Paper III: Day-care centers

At each inclusion, the parents answered a form of baseline demographics, household characteristics and medical history.

The children underwent a standardized clinical examination by one out of four pediatricians, who classified the children into three groups based on clinical findings: 1. No RTI with normal findings, 2. Mild RTI with discrete signs of rhinitis, pharyngitis, simplex media otitis or secretory otitis, and 3. Clear RTI with significant signs of rhino-pharyngitis, tonsillitis, purulent media otitis or auscultatory findings from the lower airways.

Respiratory secretions, microbiological analyses and blood samples

Nasopharyngeal swab

Flocked swabs (Copan Italy) were used to collect respiratory secretions from nasopharynx (NPS) from children in day care and the follow-up samples in HMPV-infected children enrolled for an analysis of HMPV shedding time (Papers I and III). The swabs were placed into a 3 ml transport medium (UTM-RT, Copan Italy). A flocked swab is comprised of a solid molded plastic applicator shaft with a tip, in which the procedure of collecting respiratory secretion is rather gentle, and therefore an advantage in maintaining a good compliance with repeated sampling.

Nasopharyngeal aspirate

Nasopharyngeal aspirates (NPA) were collected from children with acute RTI at admittance or the day after, and during the general anesthesia in the control children at the DP (Papers I-II). The aspirates were placed in a standard virus transport medium without antibiotics.

Microbiological analyses

Respiratory samples were cultured in cell lines (Papers I-II), with the exception of samples from children in day care (Paper III), and all were analyzed by nucleic acid detection tests (Papers I-III). The secretions were analyzed at the Department of Medical Microbiology, St.

Olav's Hospital: the analyses were carried out in the daily laboratory routine and performed within 24 hours after sample collection. However, for the samples from children in day care, the PCR- testing was done within 3-5 days after collection, due to a priority given to analyze samples from patients at the hospital (Paper III).

Nucleic acid detection

The detection of respiratory pathogens was done using in-house TaqMan real-time PCR and semi-quantitative results were based on the cycle threshold value (Ct value). PCR panels included analyses for HAdV, HBoV, HCoV (OC43, NL63, 229E), HEV, HPeV, HMPV, influenza virus A and B, PIV 1-4, RSV and HRV, *Bordetella pertussis, Chlamydophila pneumonia and Mycoplasma pneumonia* (182). The PCR test for HMPV was based on the N gene (46). In recent years, Ct values >40 were regarded as virus-negative, which was applied for the respiratory samples collected from children in day care (Paper III). Previously, Ct values >42 were regarded as virus-negative, which was applied for other respiratory samples (Papers I-II). PCR virus-negative samples were encoded with a Ct value ≥42.1 for the HMPV shedding analyses only (Paper I). A few children had both HMPV and RSV in the NPA, and were excluded from the analysis of comparison for HMPV-and RSV-infected children (Paper II), but were included in the HMPV-group when assessing the viral occurrences and hospitalization rates (Paper I). The results in Papers I-III are based on the PCR tests if not otherwise specified.

Viral culture

The majority of the respiratory samples were cultured in cell lines (Papers I-II). The following cell lines were used for viral cultures: LLC-MK2, human embryofibroblasts and human malignant glioma cells (TMG-1).

HMPV genotyping and phylogenetic analysis

HMPV-positive specimens were genotyped by real-time PCR and DNA sequenced using primers targeting the F gene of HMPV (22) (Papers I-II). A 527-bp amplicon was sequenced using a Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequences were analyzed on an ABI 3130XL (Applied Biosystems), while genotypes were determined by comparing sequenced data with the nucleotide BLAST database (www.ncbi.nlm.nih.gov/BLAST/). Some of the nasopharyngeal aspirates were not typeable due to a low viral load and others were not available. The initial genotyping was performed in December 2015, and the results were included in Paper II. In

February 2017, the sequenced data was again compared with the BLAST database, and the results were included in Paper I.

Phylogenetic comparisons of F gene sequences of 169 isolates from patients and 36 GenBank sequences representing each of the described HMPV genotypes (A1, A2a, A2b, B1 and B2) were performed. Multiple sequences were also aligned using the MUSCLE and Clustal W software. Phylogenetic analysis was inferred using the Neighbor-Joining method, with evolutionary distances calculated by the Tamura-Nei method using the Geneious v.9.0.2 software (Paper I).

Blood samples

Blood samples were collected to measure the concentration of C-reactive protein (CRP) in mg/L and white blood cell count x 10^9/L (Paper II).

Definitions

In Paper I, a season was defined as the beginning of August to the end of July the following year. An epidemic was the time between the onset and offset month during one season. The onset month was the first of two consecutive months when the monthly proportion of a virus was ≥10% positive of the total number of NPA. The offset month was the last month when the monthly proportion of a virus was ≥10% positive, preceding 2 consecutive months with <10% positive out of the total number of NPA. The peak activity month during an epidemic was the month with the highest number of children with the respective virus.

The incidence rates of hospitalization were calculated by the use of study data, ICD-10 diagnosis statistics from the patient administrative system and population data from Statistics Norway (Paper I). All data was collected per age groups and seasons, and from our study we included the number of children staying ≥24 hours with the detection of HMPV and RSV with LRTI diagnosis. The following ICD-10 codes were included: pneumonia J10.0. J11.0, J12.0-J12.9, J13-J15, bronchitis J20, bronchiolitis J21, unspecified LRTI J22 and asthma exacerbation J45-J46. Incidence rates were calculated from 7 seasons (2007/08-2013/14).

In Paper II, disease severity in children with LRTI was defined by a score. This score ranged from 0 to 4 points, and was the sum of: 1) a need for oxygen to maintain oxygen saturation ≥93% (1 point); 2) length of stay ≥6 days (1 point); 3) a need for respiratory support with a non-invasive ventilator (NIV) (1 point); 4) a need for respiratory support with both a NIV and invasive ventilator (IV) (2 points); and 5) the need for IV support (2 points).

In addition, admission to the PICU was reported as a disease severity measure. A severe disease was defined as a disease severity score ≥2 points, corresponding to- or above the 75% percentile limit among the 1,041 included children.

Statistical analyses

Frequency counts of categorical variables were compared by Chi-square Test or Fischer's Exact Test when the expected count was <5 (Papers I-III). Means of normally distributed continuous variables were compared by Student t-test (Papers I-III) or ANOVA (Paper II), and non-normally distributed continuous and ordinal variables were compared by use of the Mann-Whitney U- (Papers I-II) or Kruskal-Wallis Tests (Papers I-III). Repeated measures were analyzed by Friedman Test for ordinal variables and Cochran's Q Test for dichotomous variables (Paper I).

The duration of HMPV shedding was estimated by Kaplan-Meier analysis, in which the shedding time, in days, was estimated from the onset of symptoms to a PCR virus-negative sample (Paper I) (98). The samples were collected at a median of 4.0, 8.5 and 13.0 days after symptom onset. Four HMPV-positive specimens in the last sampling were censored.

When comparing children with HMPV and RSV infections, we stratified the analyses among those with single virus infections and those with viral co-detections (Paper II, Tables 1-3 and S1 Table in Paper II). Due to a significant age difference between the HMPV- and RSV-infected children, we also used a stratification strategy to control for age (Paper II, Table 4 in Paper II).

To analyze risk factors for severe LRTI we used logistic regression (Paper II). Severe disease (cases) was defined as a disease severity score ≥2 and controls as a disease severity score <2, and used as a binary outcome in logistic regression. Predefined factors related to the exposure and outcome were entered in a full multivariable logistic regression model. The final model was obtained by stepwise removing factors with a $P > 0.1$, and the reference category of each included factor was specified. Testing for a possible covariance of variables in the final model was performed in order to assess possible confounding, in which linear regression analyses with collinearity diagnostics and Pearson's Correlation Tests were performed. The variables in the final models were also tested for interactions, in which none were significant, and the models were kept without interaction terms. The results from logistic regression were presented as odds ratios (OR) with 95% confidence intervals (CI) and *P*-values.

The data from children in day care included variables such as children's age, viral and clinical findings, variation in seasons (4 different sampling times) and day-care section (9-10 sections) (Paper III). Generalized linear mixed-effect models (GLMM) were selected as appropriate analyses to explore both fixed and random factors related to the outcome. GLMM with logit link functions were used to explore the occurrence of HRV, HEV and HPeV in the respiratory samples, with both fixed and random factors included in the models. The "top-down" approach recommended by Diggle et al. was followed, in which the random part of the models were first determined based on the "beyond optimal model" before obtaining the minimal adequate model by selecting from among the candidate's fixed parts (183, 184). Model selection was based on the Akaike information criterion (185), with the same approach followed in order to study whether clinical findings were related to the occurrence of HRV. The response variable was the occurrence of clear RTI coded as a binary variable, with mild and no RTI as the reference category.

We also wanted to explore whether the respiratory viruses occurred independently of each other among children in day care (Paper III). The Monte Carlo simulation test described by Hope, using the algorithm by Patefield, was used (186, 187). Hope's test was further used in pairs, which of the three most common pathogens, HEV, HPeV and HRV, occurred independently of each other. In these analyses, the sequential Bonferroni Method was also used to control for Type I error rate in these three tests (188).

No imputation of missing data has been done, and the missing data has been specified within the papers. *P*-values < 0.05 (two-sided) have been considered as statically significant. Data has been analyzed by use of IBM SPSS Statistics 22, Sigma Plot 13.0 and R version 3.2.2 (189), and the R-package lme4 was used in the GLMM- modelling (190).

Ethical approval

The Regional Committees for Medical and Health Research Ethics, mid-Norway, approved all parts of the study and the CAIR biobank was approved by the National Biobank Council. In addition, the day-care study was approved by the leader of the day-care centers, the parent's committee for the day care and the chief municipal executive in Trondheim municipality.

4 Results

Paper I

The Burden of Human Metapneumovirus Infections in Hospitalized Norwegian Children

The burden of HMPV compared to RSV

HMPV was detected in 7.3% (267/3,650), RSV in 28.7% (1048/3,650), and 64.0% (2,335/3,650) had other viruses or were virus-negative in children admitted to hospital with RTI during a nearly 9-year period (Supplementary Table 1 in Paper I). HMPV varied from 2.6% to 12.4% in each of 9 seasons, an average of 7.3% per season. RSV varied from 21.3% to 39.0%, an average of 28.7%. HMPV appeared mostly from January to April (74.2%, 198/267), whereas the occurrence of HMPV from January to March in odd and even years (even year, e.g. 2006/07) was equal ($P = 0.730$). Eight out of 9 seasons had an HMPV epidemic, with a median duration of 3.5 months (Fig 4). Four seasons had HMPV peak activity in January and February, while the other four seasons had their peak activity in March or later. The winter HMPV epidemics had more children hospitalized and a longer duration than the spring-summer epidemics. RSV epidemic occurred in each of 9 seasons with a median duration of 5 months, which was longer than the median of HMPV epidemics. Additionally, the HMPV epidemics appeared before, during or after RSV.

During the entire study period, three children were hospitalized twice with HMPV infection (1-5 years in-between), elicited by different or unknown HMPV subtypes. Two of them had LRTI in both hospital periods, while the third had LRTI in the first and URTI in the second hospital stay.

In total, 900 children were hospitalized with LRTI and HMPV (n = 146) or RSV (n = 754) during seven seasons. The average (mean) hospitalization rate per season of HMPV-associated LRTI in children <5 years was 1.8/1,000 children, while the youngest children had higher rates in 2.8/1,000 children 0-11 months old and 3.6/1,000 children 12-23 months old (Table 1 in Paper I). Children with RSV had higher average incidence rates than HMPV: 9.9/1,000 children <5 years old, with highest rates among the youngest children in 27.0/1,000 children 0-11 months and 12.9/1,000 children aged 12-23 months. In children ≥24 months old, the rates gradually decreased in both HMPV-and RSV-infected children. Overall, the HMPV hospitalization rate was 5 times lower than RSV in children < 5 years old.

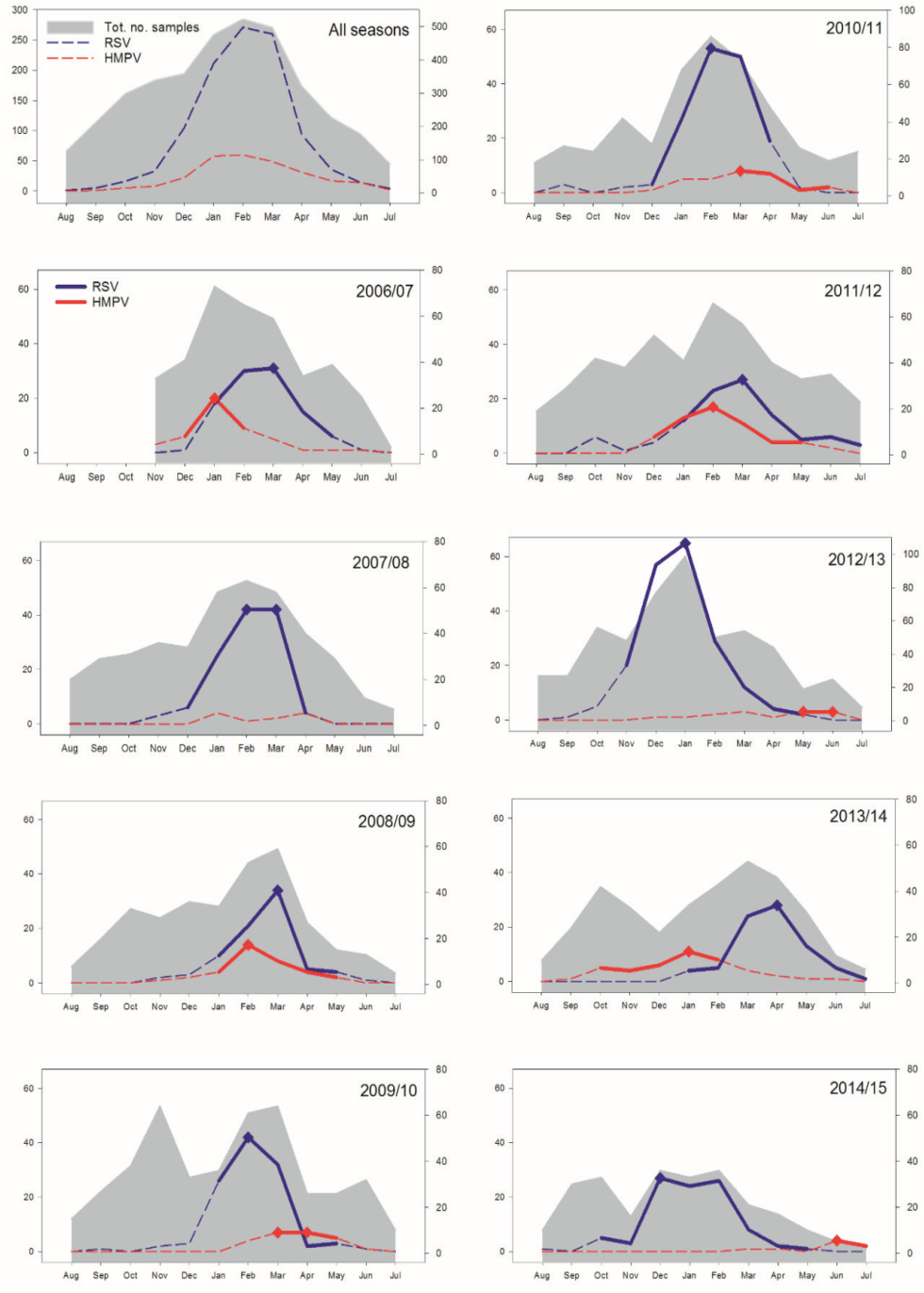
Tot. no. samples
RSV
HMPV
All seasons
2010/11
2006/07
2011/12
2007/08
2012/13
2008/09
2013/14
2009/10
2014/15
Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul

Fig 4. Detection of HMPV and RSV among children with respiratory tract infection according to month and season. The gray shade represents the total number of samples tested, with numbers at the right Y-axis. Dashed lines indicate the detection of HMPV (red) and RSV (blue) and solid lines indicates HMPV epidemic (≥10% positive) (red) and RSV epidemic (≥10% positive) (blue), with numbers at the left Y-axis. Diamonds are the peak activity month during HMPV epidemics (red) and RSV epidemics (blue). During the 2007/08 season, no HMPV epidemic occurred and therefore no peak activity month is marked. The peak activity month during an epidemic was the month with the highest number of children with the respective virus. HMPV indicates human metapneumovirus and RSV indicates respiratory syncytial virus.

HMPV in asymptomatic controls and children with acute RTI

HMPV was detected by PCR in 2.1% (7/339) of asymptomatic hospital controls, with a median Ct value of 38.9, and all were HMPV-negative by culture. The median Ct value of HMPV (median 28.0) was lower, and HMPV was more frequent in children with acute RTI than in the controls (both $P < 0.001$). The median age of HMPV-infected children with RTI were lower than all controls.

HMPV genotypes and subtypes

A total of 222 out of 267 HMPV-positive samples were genotyped: genotype B was detected in 126- and genotype A in 96 out of 222 genotyped samples. Furthermore, 37 were subtype B1, 89 were B2, 12 were A2a, 80 were A2b and 4 were A2 (unassigned). No samples were positive for A1. Two or more subtypes were detected, and one or two subtypes dominated in every season (Fig 3 in Paper I). Phylogenetic analyses showed that several strains circulated in each season, but no clusters or new strains were detected during the 9-year period.

Shedding of HMPV

Several respiratory specimens were collected from each child of the 32 included children, for a total of 93 samples. Samples were collected regularly until virus-negative. The Kaplan-Meier analysis estimated that 50% (median) and 100% of 32 children were PCR-negative after 13.0 and 28.0 days, respectively, from the onset of symptoms, with the shedding time varying from 6.0-28.0 days (Fig 4 in Paper I). The NPA taken at admittance had a median Ct value of 23.8, and 84.4% (27/32) of the samples were culture-positive. The first follow-up samples had a median Ct value of 34.7, and only 15.6% (5/32) were still culture-positive. The second follow-up samples had a median Ct value ≥42.1, with the value encoded for virus-negatives, and none of the 20 samples were culture-positive. The median Ct values gradually

increased (the viral loads were gradually reduced), and the rate of culture-positive samples gradually decreased from admittance to the first and second follow-up samples (both $P < 0.001$), and all children gradually improved.

Paper II

Comparing Human Metapneumovirus and Respiratory Syncytial Virus: Viral Co-detections, Genotypes and Risk Factors for Severe Disease

Children with LRTI and HMPV

The 171 HMPV-infected children had a median age of 17.2 months. Nearly every fourth child was born premature, and every third child had ≥1 chronic disease. Sixty-nine children had bronchiolitis (40%) and 61 had pneumonia (36%), while obstructive bronchitis, asthma exacerbation and unspecified LRTI were less common. The majority received inhalations (91%) and oxygen (60%), whereas fewer received antibiotics (39%). The median peak CRP level was slightly elevated at 35 mg/L, while the median hospital stay was 4.0 days and the median severity score was 1.0. HMPV was detected as a single virus in 106 (62%), while 65 (38%) had HMPV with one or more viruses co-detected (HRV (n = 27), HEV (n = 22), PIV (n = 9), HBoV (n = 8), HAdV (n = 6), HPeV (n = 3) and influenza viruses (n = 4)). All baseline characteristics, presence of comorbidities, clinical findings, diagnoses and disease severity measure appeared at similar rates among children with HMPV single-virus infection and those with HMPV co-detection. Hence, the disease severity score was equal.

HMPV genotypes were available from 147 out of 171 children. Genotype B was most frequent in 80 NPA, of which 26 were B1 and 54 were B2. Genotype A was detected in 67 NPA, of which 12 were A2a, 28 were A2b and 27 were A2 (unassigned). No subtype A1 was found. There were no differences in infections elicited by genotypes A and B, and the disease severity score was equal. When comparing subtype B1 vs. subtype B2, and A2a vs. A2b vs. A2 (unassigned), both as single-virus infections and co-detections, no differences were found in the median disease severity score. The analyses reported in the thesis and Paper II, were carried out again after the new genotyping in February 2017, in which the new genotyping yielded the same conclusions (data not shown).

Children with LRTI and RSV

The 859 RSV-infected children had a median age of 7.3 months, 14% was born premature and 17% had a chronic disease. Bronchiolitis was the most common diagnosis in nearly two-thirds, and approximately one-fifth had pneumonia. The majority received inhalations (96%) and oxygen (63%), whereas fewer received antibiotics (25%). The median peak CRP was 19 mg/L, while the median hospital stay was 4.0 days, and the median severity score was 1.0.

RSV as a single-virus infection appeared in 540 (63%) children, while 319 (37%) had RSV with other viruses co-detected (HRV (n = 178), HEV (n = 63), HPeV (n = 54), HBoV (n = 53), HCoV (n = 46), HAdV (n = 28), PIV (n = 12) and influenza viruses (n = 11)) (Shortcoming: the number of HEV lacked in Paper II). Some differences were detected in children with a single-virus RSV and in those with RSV with co-detection, whereas the need for oxygen, length of hospital stay and the disease severity score did not differ between the two RSV groups.

Children infected with both HMPV and RSV

Eleven children had both HMPV and RSV. The median length of stay was five days, and the severity score was median 1.0. There was no difference in length of stay, need for oxygen and the disease severity score when comparing children with both HMPV and RSV (n = 11) with the groups of children with HMPV (n = 171) and RSV (n = 859).

Comparison of children with LRTI due to HMPV and RSV

First we compared single-virus-infected children with HMPV (n = 106) and RSV (n = 540), in which the HMPV-infected children were older (14.7 months vs. 5.4 months, $P < 0.001$), more often born premature and more often having chronic diseases than RSV-infected children. More children with HMPV infections had pneumonia (36/106, 34%) than the RSV-infected children (99/540, 18%), while bronchiolitis was more common in RSV (383/540, 71%) than HMPV (42/106, 40%) (Table 2 in Paper II). The disease severity score was equal in HMPV- and RSV-infected children (Table 3 in Paper II). However, when we controlled for age, there was no difference in the distribution of diagnoses, while the disease severity scores in the two virus groups differed (Table 4 in Paper II). Among children <6 months old, nine out of 10 children with HMPV, as well as those with RSV, had bronchiolitis. In the youngest age group (<6 months old), children with an HMPV infection had a milder disease than RSV, while the pattern was the opposite in the age group of 12-23- month old children (Fig 5).

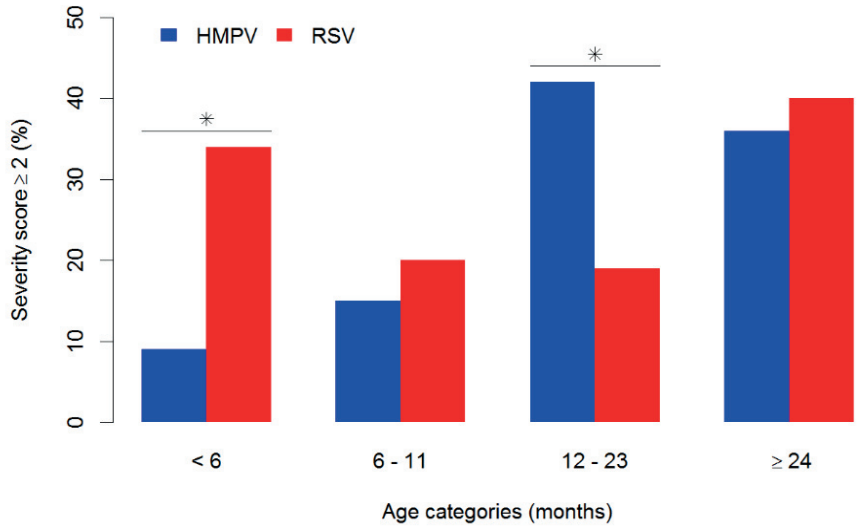

Fig 5. Proportions of children (%) with severe lower respiratory tract infection, severity score ≥ 2, among single-virus-infected children with HMPV (blue) (n = 106) and RSV (red) (n = 540), according to age categories. Asterisk indicates significant differences ($P < 0.05$).

We also compared children with co-detection of other viruses, in addition to HMPV (n = 65) and RSV (n = 319). In these groups, we also found a difference in age (HMPV: 18.5 months vs. RSV: 12.5 months, $P < 0.001$), while there was no difference in diagnoses, and the disease severity score was equal.

Risk factors for severe disease due to HMPV and RSV

In the logistic regression analyses, we found that age was associated with disease severity (Table 5 in Paper II). In HMPV-infected children, the age groups of 12-23 months (OR = 3.01, $P = 0.067$) and those ≥24 months (OR = 3.97, $P = 0.021$) were associated with the highest risk for severe disease, whereas in the youngest age group (<6 months) RSV-infected children had the highest risk (OR = 1.58, $P = 0.035$). Prematurity and ≥1 chronic disease were associated with increased risk in both HMPV- and RSV-infected children. A high viral load was associated with a higher risk for severe disease in RSV-infected children only, and not in those with HMPV. Viral co-detection was not associated with an increased risk for severe disease, and not included in the final models for HMPV and RSV. Finally, HMPV genotypes

were not included in the predefined model, but were analyzed with logistic regression, but were not associated with disease severity.

Paper III

Respiratory Virus Detection and Clinical Diagnosis in Children attending Day Care

Viral findings

NPS were collected in 343 out of the 368 inclusions (Fig 1 in Paper III). Taken together, 43% (149/343) of NPS were virus-positive, with the rate varying from 34% (25/74) to 56% (55/99) at each study visit. There was large variation in the virus detections during the four visits, and only HEV, HPEV and HRV were detected in all visits (Fig 6).

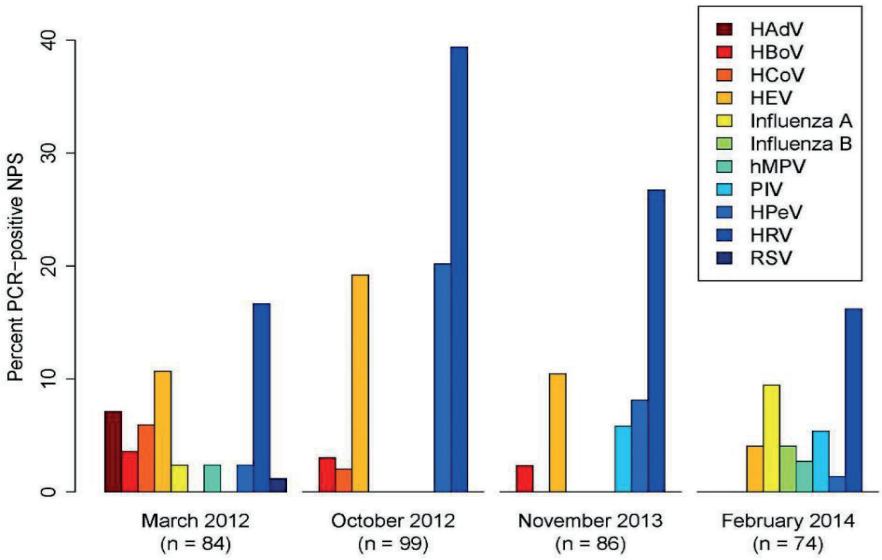

Fig 6. Viral findings at each study visit. Percentage of nasopharyngeal samples that was positive for each of 11 virus types (genotypes of HCoV and PIV not shown). Nasopharyngeal samples were collected at four different sampling times.

HRV was detected in 26% (88/343), HEV in 12% (40/343) and HPeV in 9% (30/343), whereas ten other viruses were each detected in ≤3%, including HAdV (n = 6) and HBoV (n = 8). One virus was detected in 31% (106/343) of the NPS and two or more viruses in 12% (43/343). The Monte Carlo stimulation test showed that NPS with ≥2 viruses were more frequent than expected if the viruses were randomly and independently distributed among NPS, while single-virus samples were less frequent than expected. Thus, there was a general tendency that viruses occurred together in the NPS, although this tendency was not due to an

uneven occurrence of viruses among day-care sections and sampling times. The picornaviruses were in particular involved in coinfections (≥2 viruses). The GLMM analysis showed that the occurrence of HEV varied randomly among combinations of sections and sampling times; this means that the occurrence of HEV varied from zero to approximately 80% between section, but it was not the same sections that had a low or high prevalence each time (Fig 3A in Paper III). HEV occurred mostly in the sections of young children. HPeV varied also among sampling times and occurred mostly in the young children (Fig 3B in Paper III). The occurrence of HRV varied also randomly among combinations of sections and sampling times, while HRV was common in sections of both young and older children (Fig 3C in Paper III).

Clinical findings- and association between clinical and viral findings

In 355 of the 368 inclusions, a clinical examination was performed. NPS were collected from 331 of the examined children, among whom 24% (79/331) had clear findings of an RTI, 41% (135/331) had mild findings and 35% (117/331) had normal findings. Children with a clear RTI were younger than those with a mild RTI and no RTI (Table 1 in Paper III). Seventy percent (55/79) of children with a clear RTI were virus-positive, compared to 41% (55/135) in those with mild findings and 30% (35/117) in those without an RTI. HRV was most frequent in all clinical groups. The GLMM analysis of the occurrence of a clear RTI included positive effects for the occurrence of HRV and negative effects for children's increasing age, together with random effects for a combination of day-care sections and sampling times. Only 14 children had HMPV (n =4), RSV (n = 1) or PIV (n = 9), in which 11 had mild or clear signs of an RTI. Those with HMPV had a mild (n = 3) or clear RTI (n = 1) findings, in which 2 of the NPS in children with a mild RTI had either HCoV or HRV co-detected.

Parental reported symptoms

Based on information collected from the caregivers, 84% (54/64), 65% (74/113) and 45% (40/89) of the children with clear, mild or no clinical signs of RTI had respiratory symptoms at the examination time or two weeks prior. Among the 55% (49/89) without reported symptoms and with normal findings, still 24% (12/49) had one or more viruses: HCoV (n = 1), HEV (n = 2), HPeV (n = 3) and HRV (n = 8).

5 Discussion

The main findings of the thesis are that HMPV frequently appears in Norwegian children, and in regular winter and spring-summer epidemics. HMPV has a substantial disease burden, although RSV is associated with a five times higher hospitalization rate. Clinically, severe HMPV infections manifested independent of viral co-detections and HMPV genotypes. Overall, the clinical manifestations in HMPV- and RSV-infected children were relatively similar, and the risk factors for severe infections were related to the child's age, a history of prematurity and chronic disease. The low HMPV detection rate among hospital controls and children in day care, a short HMPV shedding time, and the fact that viral cultures were all HMPV-negative in the controls, support the epidemic nature of HMPV. On the other hand, many apparently healthy children in day care were positive for picornaviruses, which are noteworthy, but may not be surprising since children often have RTI.

The burden of HMPV compared to RSV

The epidemiology of HMPV has been described before in many hospital-based studies, but only a few were based on a comparable long study period. Over 9 seasons, we found that HMPV was detected in 7.3% of all children admitted with RTI during the entire period, but varied from 2.6% to 12.4% per season. Most previous studies from the Northern Hemisphere found quite similar figures and seasonal variations (2, 3, 57, 64, 77, 107). In the present study, RSV was detected more frequently than HMPV, as reported from others (3, 63, 64, 77).

HMPV appeared mostly from January to April, and regularly caused outbreaks. The occurrence of HMPV from January to March in odd and even years was equal, in contrast to observations from southern Europe, with alternating high HMPV activity in winter and spring-summer every other year (57, 84). There may be several reasons for this difference, and we speculate as to whether this may be related to the climate differences (191). HMPV epidemics peaking in the winter months had more children hospitalized and a longer duration than spring-summer epidemics. The RSV epidemics peaked mostly in January to March as observed in others countries with a temperate climate (63, 83, 84), and appeared before, overlapping with or after HMPV (63). Others have reported that HMPV may appear anti-cyclical with RSV (64, 83, 84) Overall, in present study, HMPV and RSV mainly appeared during winter and early spring and the prevalence differed among seasons, as observed from other countries with the same climate (64, 83). As compared with other viruses, the detection

rate of HMPV in our data is at somewhat similar rate as for influenza viruses and PIV in other studies (64, 83).

In the present study, the average hospitalization rate per season of HMPV-related LRTI was 1.8/1,000 children aged <5 years old, based on data from 7 seasons. Children in the youngest age groups had higher rates. Our estimates differed and were higher than three US studies, reporting estimates from 1.0- to 1.2/1,000 children <5 years old (2, 67, 80). Two European studies reported HMPV-associated incidence rates somewhat comparable with ours. A study from Spain (60), based on 3 seasons, reported that 2.6/1,000 children <3 years old were hospitalized, and in a single season study from the UK the rate was reported to be 1.3/1,000 children <6 years old (91). Three of these studies included children with acute respiratory illness or fever (2, 60, 67), and one had an even broader inclusion (91). We used a more strict definition for severe HMPV infection, including only children with a hospital stay ≥24 hours and with a LRTI diagnosis, which may explain that our estimate is higher than 2 from the US (2, 67). Compared to our prospective, the third US study (80) was retrospective, which may explain the differences observed. Moreover, our estimates are based on 7 seasons which may also explain some of the difference observed in relation to studies based on 1-3 seasons (60, 67, 91). Overall, we believe that our estimate of the HMPV-related hospitalization rate has provided a reliable medical statement of the disease burden of severe HMPV infections in Sør-Trøndelag County. The differing results among studies may be explained by differences in study design (i.e. inclusion criteria, age groups, prospective/retrospective) and duration.

The hospitalization rates of children with RSV-related LRTI in our study were in line with findings from previous Norwegian (156), European (157, 192) and American studies (158, 162), thereby confirming that HMPV causes hospitalization less often than RSV in Europe and the US.

We detected all known HMPV subtypes, with exception of subtype A1, with subtype B2 being the most frequent subtype over the entire period. In line with other studies (4, 23, 57, 65, 88-90), the distribution of subtypes exhibited great seasonal variation. In every season one or two subtypes dominated, and at least two subtypes circulated. Additionally, the majority of previous studies (4, 23, 57, 65, 88, 89) and the present study, did not detect A1 or a very few, with the exception of several cases in Italy in 2009/10 (79). The phylogenetic analyses showed many, but well-known strains, circulating during the entire 9-year study period, but no clusters or new strains. On the other hand, the evolutionary dynamics of HMPV

have demonstrated high substitution rates, as other RNA viruses (17, 134, 193). Hence, phylogenetic analyses in the population and the antigenic variation of HMPV in humans (17) is important for the development of future vaccines and antivirals (23).

As indicated by the hospitalization rates, the incidence of severe HMPV infection decreased by age. In addition, only a few children were hospitalized with recurrent HMPV infections elicited by unknown or different HMPV subtypes. Previous research has shown that most children became seropositive during the first 5 years of life (1), while data from experimental studies suggest that certain HMPV subtypes may not stimulate an adequate immune response in all cell types (123). Our clinical data support that healthy children usually develop a robust immunity against most HMPV subtypes during childhood, which is in line with others (80). On the other hand, outside a hospital setting, others have shown that HMPV may still cause recurrent mild RTI in children (38) and adults (13). Moreover, children (113) and adults (114) with impaired immunity may be prone to severe HMPV infections, even with a high seroprevalence at all ages (122).

HMPV in asymptomatic controls and the HMPV shedding time

Only a small percentage of the asymptomatic controls in our study had HMPV detected by PCR with high Ct levels, thereby corresponding to low viral loads, though all were virus-negative by culture. Among the group of children with HMPV infection, which were sampled with repeated specimens, most had low Ct values (high viral loads) and a high rate of positive cultures initially. During the disease, these children improved clinically, viral loads gradually decreased, and all became virus-negative by culture after 13 days. Despite these changes, half were still virus-positive by PCR test after 13 days, and all were negative after 28 days. Taken together, we believe that these observations along with observations from others (2, 94-97), support the hypothesis that a positive PCR test for HMPV in healthy children is unlikely to indicate an asymptomatic infection, so we speculate whether this rather indicates the presence of a small amount of viral nuclei acids after a previous HMPV infection. Others have also shown that the HMPV viral loads rapidly dropped along with clinical improvement (97). Based on our estimates of the HMPV shedding time, along with data from others (94-97), it seems that the HMPV shedding time may be quite similar to other epidemic viruses, such as RSV and influenza virus in 2-3 weeks (94, 98, 99).

Clinical manifestations in children with HMPV and LRTI

It has been discussed whether viral co-detections increase the disease severity or not. In the group of HMPV-infected hospitalized children with LRTI in the present study, we detected more than one virus by the use of PCR tests in 38% of the children. All baseline characteristics, including rates of prematurity and chronic diseases, clinical manifestations and clinical courses were equal to those with a single HMPV infection and HMPV with viral co-detection. Our study is large and population-based, and along with observations done by others (5, 11, 71, 117), it seems evident to conclude that viral co-detections in HMPV-infected children usually have no cumulative effects to that of HMPV alone. In some early HMPV-studies, it was reported increased disease severity in children with HMPV/RSV-coinfections (111, 112). Our data showed that co-detections with both HMPV and RSV were rare, and was not associated with increased disease severity in our population. Another large study reported higher rate of such co-detections than we found, while similarly, no increase in the disease severity compared to HMPV alone was observed (11).

We detected a broad spectrum of HMPV genotypes causing infections with quiet similar clinical manifestations and outcome. Hence, our findings confirm results from previous studies with smaller sample sizes (5, 70, 72, 89, 118). However, others genotyped HMPV-positive children <3 years of age, finding that either genotype A (11, 69) or B (37) was associated with an increased disease severity during the 3 to 4 study years. Two of the studies had differing disease severity measures, either as oxygen need (11), or oxygen need, PICU attendance or a hospital stay <5 days (37), and two studies had a sample size <70 children (37, 69). The differing results from our study may be explained by the differences in age of the included children, sample size and because the outcome “severe disease” in two of the other studies probably included less ill children than in our study. Moreover, naturally occurring genotype variation over short time intervals may also increase the risk of random findings. After the new genotyping of our data in February 2017, we found similar results as presented in the thesis and in Paper II.

HMPV-and RSV-infected children with LRTI

The most prominent factor differing between HMPV- and RSV-infected children with LRTI was the difference in age distribution, which has been observed before (37, 71, 105, 106, 194). In addition, we confirmed findings from previous studies that more HMPV-infected children were born preterm (37, 106) and had a chronic disease (2, 104). HMPV and RSV

caused a relatively similar spectrum of LRTI types. In the single-virus groups of HMPV and RSV, bronchiolitis was the most common diagnosis in both viruses. However, HMPV-infected children apparently had pneumonia more often. The disease severity score was also equal in single-virus groups. Even so, these findings were confounded by age. After controlling for age, no difference in the distribution of diagnoses was found, while the disease severity score differed. HMPV infection was associated with a milder disease than RSV among children aged <6 months, whereas the pattern was the opposite in the age group of 12-23-month-old children. Others have reported a similar disease severity in children with single-virus infections of HMPV and RSV (71, 74, 172), and two of the these three studies also used age-matching (74, 172). However, their age-matching included children in a very wide age-range and not in narrow age-groups as we did. On the other hand, a more severe disease in RSV than HMPV has also been reported (37), but in this study coinfections were not excluded and no age adjustment was performed. Taken together, we conclude that age was strongly related to disease severity, and the age effect differed among single virus HMPV- and RSV-infected children. One possible explanation for that might have been that neonates attain a higher concentration of maternally derived protective antibodies against HMPV, as compared to RSV, during pregnancy. Nonetheless, a study measuring HMPV and RSV antibodies in infancy did not support this hypothesis (40). In general, clinical manifestations in children with airway infections are related to the net effect of physical and genetic factors, as well as viral- and immune-mediated reactions in the maturing child, which are strongly correlated to the child's age (36, 195).

We found that high RSV viral loads, but not high HMPV viral loads, were associated with severe disease. Thus, it may be tempting to suggest that RSV is a more potent virus than HMPV among infants, and that RSV more than HMPV is a virally driven disease. Others have published data supporting a similar assumption of RSV (51). In accordance with our findings, others found that the HMPV viral load was not associated with an increased disease severity among hospitalized children (118, 119). By contrast, hospitalized (inpatients) patients had higher HMPV viral loads than outpatients, and children with LRTI had higher HMPV viral loads than URTI (118, 121). Hence, it seems that HMPV viral loads may be related to disease severity to a certain degree, but not among those with the most severe disease.

Several of the previous studies on the risk factors for severe HMPV infection focused on children younger than 2-3 years of age (11, 37, 106), high-risk patients (106) or for children admitted to a PICU (110). Additionally, others were retrospective (104, 109) and

some had a limited number of HMPV-infected children included (37, 109). The disease severity has also been defined by the use of various outcome variables, and only a few studies have directly compared HMPV and RSV (37, 106). We included a population-based sample of all children aged <16 years who were hospitalized with acute RTI, although the majority were aged <5 years. We used a compound disease severity score combining several outcome measures, and rather rigorously defined severe disease. We confirmed that independent risk factors for both severe HMPV and RSV infections were the presence of chronic diseases and a history of prematurity. Children aged 12-23 months, and those aged ≥24 months had an increased risk for severe HMPV infection, while among RSV-infected children the youngest (<6 months old) had the highest risk. Overall, the risk factors for severe infections with HMPV and RSV were relatively similar, and related more to the child (age, a history of prematurity and chronic disease) than to viral co-detection. Because of this, our data confirm the findings from other studies that particular age groups, prematurity and the presence of chronic diseases independently increase the risk of developing severe LRTI among children with HMPV infection (2, 11, 37, 104, 106, 109, 110) and RSV infection (37, 106, 162-165).

HMPV and other respiratory viruses in day-care children

We detected one or more respiratory viruses in 4 out of 10 children attending day care. One-fourth had clear signs of an ongoing RTI, and 4 out of 10 had milder signs of RTI. Picornaviruses were the most frequently detected viruses, whereas HMPV and RSV were rare. Rhinovirus appeared most in day-care children, but enterovirus and parechovirus were also common. Combinations of day-care sections (younger or older children) and sampling times (season) were important factors determining the occurrence of picornaviruses. At any given sampling time, there was a large variation in the frequencies of the three picornaviruses among various sections, and for most sections there was a large variation at different sampling times. These observations may be related to the fact that most respiratory viruses are epidemic and easily spread among children who are cared for in separate sections (196). This phenomenon was most common in sections for the youngest children, who are particular known to challenge good hygiene.

There was a general tendency that viruses occurred together, independent of the influence of sections and sampling times. Similarly, others have reported that some virus combinations may appear more frequently and that co-infections with viruses may not be random in children (93, 175-177).

Only a few children had HMPV and RSV, and we speculate whether these viruses more often cause severe disease and sick leave from day care. Moreover, some of the visits in day care were performed during HMPV- and RSV epidemics observed in hospitalized children (Fig. 4). As a result, both HMPV and RSV circulated in the community, while seldom being detected in day-care children.

As shown by others, children with an HRV-positive NPS had increased probability of a clear RTI and HRV likely being the cause of many RTI in children outside of a hospital (87, 141, 144, 175). However, we also detected HRV in children without clinical findings of an RTI, while only a few samples were positive for HBoV and HAdV in the three clinical groups. As for HRV in asymptomatic children several mechanisms have been described**.** HRV may persist as positive up to several weeks after recovery from an RTI, because of a long shedding time (102, 175), while others have shown that a minor fraction of HRV infections may be asymptomatic (197). Recent data has shown that HRV-positive children with and without symptoms developed different immune responses, which supports that HRV detection may not always indicate a symptomatic HRV infection (198). HBoV and HAdV have previously often been detected in healthy and asymptomatic children, either due to prolonged shedding or due to the re-activation of a latent infection (100, 101, 175). However, HRV and HAdV are well-known causes of RTI, and recent evidence supports that HBoV may cause an acute RTI (49, 199). We found that several children with HRV and clear signs of RTI attended day care and were apparently healthy, which could suggest that HRV in other cases may also cause mild changes that are hard to detect at all.

In summary, the viral occurrences in day-care children were related to age, clinical signs of RTI, location in day care and sampling times (season).

Viral co-detection

I have previously discussed whether viral co-detections were related to the disease severity in children with severe RTI. Moreover, high rates of viral co-detections were even found in day-care children and asymptomatic controls. In the group of asymptomatic children, HMPV, RSV and influenza viruses were seldom detected, while the picornaviruses were frequent and viral co-detection occurred in 35% (unpublished data Moe et. al., see Table 1 in Appendices). In the day-care children, picornaviruses dominated and were often involved in viral co-detections. Among children with LRTI, the co-detection rates were quite similar in HMPV- and RSV-infected children (38% and 37%, respectively), and the disease severity was not

related to viral co-detections. Even in these groups, the co-detected viruses were mostly picornaviruses, whereas influenza viruses were rare. Hence, viral co-detections occurred in all cohorts and often involved one or more of the picornaviruses. As previously mentioned, others have shown that viral co-detections are frequent in both asymptomatic (48, 49) and symptomatic (48, 50) children, as we also observed. As previously stated, more than 100 HRV serotypes exist. Our PCR test for HRV detects rhinovirus types A, B and C, all with many subtypes, and a positive test result may therefore indicate various rhinoviruses each time. As a consequence, we do not know which subtypes of HRV that occurred in the three cohorts. Our research group, has previously shown that HBoV-DNA is common both in children with RTI and controls, whereas HBoV-mRNA testing, indicated the presence of active viral replication and occurred mostly in infected children (199). Data from Paper I, showed that HMPV was detected in a few cases by PCR in asymptomatic controls, although all HMPV cultures were negative. Hence, it is likely that several mechanisms may explain frequent viral co-detections with PCR. First, it may reflect an asymptomatic infection, as is has been shown for rhinovirus (48, 197, 198). Secondly, it may indicate prolonged shedding, as for HAdV (101) and HBoV (100), and finally, a positive PCR test may also indicate an early phase of a new infection (10). However, we cannot exclude the possibility that viral co-detection might also indicate two simultaneous infections, although it has been shown that viruses may compete for the resources of target cells, so that viruses with high growth rate will out-compete viruses with lower growth rate during infection (200). Furthermore, to make it even more complex, it is well-known that viruses and bacteria also interact during RTI (201) and even in healthy children (93).

Strengths and limitations of Papers I-III

Papers I-II

It is a strength of the studies, that they were population-based and prospective, and that we enrolled children of all ages in the same county in mid-Norway, as well as to the only existing pediatric hospital in this region during a nearly 9-year long period. Furthermore, 82-89% of all children admitted to hospital with an acute RTI who had a nasopharyngeal aspirate collected were included in the main study cohort, which is also a strength of present study. It is also an advantage that we used the same PCR tests and viral cultivation methods during the entire period. By using a broad panel of PCR tests, we could thoroughly examine viral co-detections. Nevertheless, it may be a limitation that bacterial co-detections were not

considered, but most children had low or moderately increased CRP values. Moreover, during the entire study period almost all Norwegian children received conjugated pneumococcal vaccines, which reduced the incidence of pneumococcal infections (202). Another limitation may be that clinicians were not blinded for the NPA results, in which diagnostic and work-up biases could have affected our results, and the patients were not treated after a study protocol. Due to practical challenges, including children 24 hours a day, some were included after passive consents. The baseline characteristics of these children were abstracted from the hospital medical records, as a substitute for the questionnaire usually filled out by caregivers. Hence, this may have affected some of the basic information (e.g. siblings, day care), since medical files often are quite short and misses important data for a study.

Some HMPV-positive samples were not genotyped, and some were unassigned A2. After the new genotyping in February 2017 only a few unassigned A2 remained, due to a reclassification into A2a or A2b. However, the clinical data of HMPV genotypes A and B, and disease severity scores in the subtypes reported in Paper II, was reanalyzed, and yielded the same conclusions. The A1 subtype might have been present in some of the non-genotyped samples, and the pattern of circulating HMPV subtypes might have been even more heterogenic than described.

The use of a control group is a strength of the cohort study, because we could compare the viral detection rates between children with clinical signs of infection with some who were asymptomatic. However, it may be a limitation that the controls were generally older than children admitted with an RTI, they were sampled during anesthesia and they were not clinically examined in order to confirm the absence of any RTI. Controls were neither contacted after sampling to assess whether subsequent RTI symptoms had occurred. But all these factors might have contributed to higher viral detection rates among controls.

For the HMPV shedding study, the children were initially sampled with NPA, while the follow-up samples were taken with NPS, in which differing sampling techniques may have been a disadvantage. Still, a recent study showed no difference in viral detection rates and viral loads between NPA and NPS, which were collected simultaneously by using real-time PCR in children (203).

Paper III

In the day-care study, we managed to include 76% of day-care children. To describe complex microbiology, we collected seasonal samples in the fall, winter and early spring with a

prospective and cross-sectional study design. The day-care section was also considered, and turned out to be an important predictor of virus occurrence. Nasopharyngeal sampling is unpleasant and challenging to perform in apparently healthy children outside health institutions. However, we managed to collect samples from more than 90% of the inclusions. It is also an advantage that pediatricians clinically examined the children, and their findings were used in the classification. Most studies on respiratory viruses and RTI in day-care settings have relied on parental information of children's symptoms. Ideally, more samples from each child, using a longitudinal design, might have had advantages over the cross-sectional approach. The clinical entities of a mild and clear RTI, which were used in the classification, have not been validated. Each study visit was performed 3-12 months apart, which is a long time from an epidemic and clinical perspective, so therefore, the analyses were not adjusted for repetitive data. Yet, it may be that some children with chronic diseases in upper or lower airways were more prone to respiratory viruses or had a latent viral infection, which may have affected the results.

Implications for public health and future studies

Current studies have shown that children with a chronic diseases and a history of prematurity had high risk for developing severe infections during the hospitalization period. In addition, the youngest infants had a high risk for a severe RSV infection, while somewhat older children for a severe HMPV infection. Hence, future HMPV and RSV vaccines for children with comorbidities are highly important, as well as the timing of vaccination, in the early infant period, or even in the mother.

Only a few studies have examined the relationship between early childhood severe HMPV infections and the subsequent development of asthma. This is an important topic and needs further investigation, and therefore, the CAIR group has recently started a follow-up study of children hospitalized with first time HMPV in our cohort. Furthermore, in order to reduce the high prevalence of asthma, future vaccines may also be important in this perspective.

Due to the increasing level of antibiotics-resistant bacteria, the use of antibiotics in childhood respiratory tract infections is often discussed. However, respiratory viruses are the most common pathogens in respiratory tract infections (142, 204, 205), but several of the previous studies of HMPV infections in hospitalized children reported that 50% or more received antibiotics (37, 105, 118). In our study, we revealed that 39% and 25% of children

with severe HMPV and RSV infections, respectively, received antibiotics. For this reason, we may have used antibiotics more rarely than others, but a future reduction in the use of antibiotics is still important to achieve. Hence, researchers and clinicians have an important task to provide information and guidelines in order to influence the public health care system and thereby the public health.

The day-care study showed that the spread of viruses within children in separate sections has occurred. In the Norwegian society, it has become common to keep children in rather large day-care units, so-called “basebarnehage” (open-plan day-care center), in which 30-40 young children have a common area. This is considered to be cost-effective due to low building costs and fewer employees per child. However, this large-unit system involves high densities of children, and even higher than in the day-care centers we visited in our study. Such large units and high densities may lead to more spread of viruses among children. Consequently, the children and employees may be more often sick, also imposing sick leave on the parents. This health perspective and these socioeconomic costs are not well understood, but may have considerable impact on individuals and society. A future multidisciplinary research project, including clinicians, social economists and researchers in public health, could evaluate the day-care centers with low, middle and high densities among children, in order to find the best solution for children, parents, employees and the society.

6. Conclusion

HMPV occurred in regular epidemics, and is a common cause of severe RTI in Norwegian children. HMPV is associated with a substantial disease burden, although the hospitalization rate was five times lower than RSV. Clinically, severe HMPV infections manifested independent of viral co-detections and HMPV genotypes. Children with severe HMPV and RSV infections had quite similar clinical manifestations and disease severity. However, age was strongly related to disease severity, and the age effect differed among single-virus HMPV- and RSV-infected children. Children with a history of prematurity and chronic diseases had an increased risk for severe infections due to HMPV and RSV. Children shed HMPV shortly, whereas HMPV appears seldom in asymptomatic children and in healthy day-care children. In contrast, the picornaviruses may be detected frequently in Norwegian children attending day-care.

7. References

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Appendix

Table 1. Number of viruses in nasopharyngeal aspirates (NPA) collected from asymptomatic hospital controls and number of NPA with viral co-detection (≥2 viruses)

NPA result	n = 339	Viral co-detection (n)
Virus negative	91 (27)	
Positive any virus	248 (73)	
Single virus	130 (38)	
Viral co-detection	118 (35)	
HAdV	25 (7)	20
HBoV	33 (10)	31
HCoV	31 (9)	20
HEV	88 (26)	70
Influenza A and B	3 (1)	2
HMPV	7 (2)	7
HPeV	31 (9)	25
PIV	39 (12)	35
HRV	163 (48)	87
RSV	11 (3)	4

Data presented as absolute numbers and percent in parenthesis, except from absolute numbers in viral co-detections.

Paper I

The Burden of Human Metapneumovirus Infections in Hospitalized Norwegian Children

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ABSTRACT

Background: The burden of severe human metapneumovirus (HMPV) respiratory tract infections (RTI) in European children has not been clarified. We assessed HMPV in Norwegian children and compared hospitalization rates of HMPV and respiratory syncytial virus (RSV).

Methods: We prospectively enrolled children <16 years old hospitalized with RTI and asymptomatic controls (2006-2015). Nasopharyngeal aspirates were analyzed by polymerase chain reaction (PCR) tests for HMPV, RSV and 17 other pathogens. We genotyped HMPV-positive samples, and assessed shedding time in 32 HMPV-infected children.

Results: In children with RTI, HMPV was detected in 7.3% (267/3,650) and RSV in 28.7% (1048/3,650). Among controls, 2.1% (7/339) had low HMPV levels detected by PCR, but all were culture-negative. HMPV primarily occurred from January to April and in regular epidemics. At least two HMPV subtypes occurred each season. The average annual hospitalization rates in children <5 years old with lower RTI were 1.8/1,000 (HMPV) and 9.9/1,000 (RSV). Among children with RTI, median HMPV shedding time by PCR was 13 days (range 6-28 days), but all were culture-negative (non-infectious) after 13 days.

Conclusions: HMPV appears in epidemics in Norwegian children, with a five times lower hospitalization rate than RSV. Low levels of HMPV is rarely detected in healthy children.

Keywords: burden of respiratory tract infections, hospitalization rate, human metapneumovirus, respiratory syncytial virus, healthy controls, virus shedding time.

INTRODUCTION

Human metapneumovirus (HMPV) causes upper and lower respiratory tract infections (RTI) in children, including severe diseases, such as pneumonia and bronchiolitis, in need of hospitalization [1-4]. HMPV is an epidemic virus that occurs in outbreaks all over Europe [5-9] and in other continents as well [10-14]. Aberle et al. [15] showed that in Austria the occurrence of HMPV had a biennial pattern with alternating winter and spring seasons of high activity. HMPV is included in the *Pneumoviridae* family with two main genotypes (A and B) and at least 4 subtypes (A1, A2, B1 and B2) [16-19]. Previous research has shown that HMPV genotypes A and B often circulate during the same season, while the dominant subtype may differ from one epidemic to the other [6, 7, 15, 19].

Although HMPV has been known for more than a decade, limited information exists about hospitalization rates associated with HMPV infection in European children. In three studies from the US, the average annual rates of hospitalization were reported to be from 1.0 to 1.2 per 1,000 children <5 years old, and higher rates were detected in the youngest [1, 20, 21]. Two European studies have reported somewhat higher rates [22, 23]. However, these studies had a limited duration, and there is a need for a population-based study covering a longer period from a European country.

In recent years, sensitive polymerase chain reaction (PCR) tests have been used to detect airways viruses, and it has been shown that RTI is often associated with the detection of nucleic acids from more than one virus [4, 24]. Still, viral co-detections may be common, even in asymptomatic children [25, 26]. It has been suggested that a prolonged viral shedding after an infection may be one explanation of subsequent co-detections in both asymptomatic and infected children [27-29]. Even so, a few studies with a limited number of patients found that HMPV may have a rather short excretion time [30, 31], which on the other hand could

explain why HMPV has been detected in asymptomatic controls less often than several other respiratory viruses [1, 3].

We aimed to study the burden of severe HMPV infections in Norwegian children, compared to respiratory syncytial virus (RSV). For this purpose, we measured HMPV, HMPV genotypes and RSV among children admitted to hospital one or more times with RTI during a 9-year-long period, and compared population-based hospitalization rates of HMPV and RSV. In addition, we wanted to evaluate HMPV in healthy children. For that reason, we assessed the occurrence of HMPV in a group of asymptomatic hospital controls, and studied the shedding time of HMPV in children with RTI.

METHODS

Study design and population

Children <16 years admitted for acute RTI with a nasopharyngeal aspirate sampled on clinical indications were prospectively enrolled at the Pediatric Emergency Department (PED) and Pediatric Departments (PD) at St. Olavs Hospital, University Hospital of Trondheim, Norway (Figure 1A). The inclusion period was from November 2006 to July 2015. Children with cytostatic and immune-suppressive treatment were excluded. During the period from June 2007 to April 2015, similarly aged children hospitalized for elective surgery were prospectively enrolled as healthy controls (Figure 1B). None of the controls were admitted for ear, nose and throat surgery, while controls with caregiver reported symptoms of RTI during the last 2 weeks or at inclusion were excluded.

The hospital is the only hospital for children in Sør-Trøndelag County in The Mid-Norway, and provides care for 58,443 children <16 years and 18,768 children <5 years of age (Statistics Norway). Informed written consents to participate were collected from caregivers to most of the children and from children ≥12 years during the hospital stay. Some children with RTI were enrolled after hospital discharge after passive consent. In addition, we enrolled some children with acute HMPV infection for analyses of HMPV shedding time. These children were sampled during the hospitalization period and weekly after discharge during home- or outpatient visits, and until the HMPV-tests turned negative. We systematically collected baseline characteristics from a questionnaire filled out by caregivers. Clinical information was abstracted from medical records, and Regional Committees for Medical and Health Research Ethics in Mid-Norway approved the study.

Clinical Classifications and Laboratory Investigations

Children admitted for acute RTI were examined and treated routinely at the discretion of medical doctors. As previously described, LRTI were categorized into five categories: bronchiolitis (children <2 years), obstructive bronchitis (children ≥2 years), pneumonia, asthma exacerbation and unspecified LRTI [4].

Nasopharyngeal aspirates (NPA) were collected from children with RTI at admittance and during the general anesthesia in the controls. NPA were placed in a standard virus transport medium without antibiotics. Flocked swabs (Copan Italy) were used to collect follow-up nasopharyngeal samples and placed immediately into a transport medium (UTM-RT, Copan Italy). All samples were analyzed at the Department of Medical Microbiology, St. Olavs Hospital, University Hospital of Trondheim, using in-house TaqMan real-time PCR assays and conventional viral cultures for 19 respiratory pathogens, as previously described [4, 32]. Semi-quantitative results from the PCR tests were based on the cycle threshold value (Ct value), with values above 42 regarded as negative. In all 222 (83%) HMPV-positive specimens were genotyped by real-time PCR and DNA sequencing by primers targeting the F-gene of HMPV [18], as previously described [4]. Some of the NPA were not typeable due to low viral loads, and others were not available. Phylogenetic comparisons of F-gene sequences of 169 isolates from patients and 36 GenBank sequences representing each of the four described hMPV genotypes (A1, A2a, A2b, B1 and B2) were performed. Multiple sequences were aligned using the MUSCLE and Clustal W software. Phylogenetic analysis was inferred using the Neighbor-Joining method with evolutionary distances calculated by the Tamura-Nei method using the Geneious v.9.0.2 software.

Definitions and Statistical Analyses

A season was defined as the beginning of August to the end of July of the following year. An epidemic was the time between onset month and offset month during one season. The onset month was the first of two consecutive months when the monthly proportion of a virus was ≥10% positive of the total number of NPA. The offset month was the last month when the monthly proportion of a virus was ≥10% positive, preceding 2 consecutive months with <10% positive samples. The peak activity month during an epidemic was the month with the highest number of children with the respective virus. Sixteen children had both HMPV and RSV in the NPA, and were included in the HMPV group. To calculate annual hospitalization (incidence) rates we used study data, ICD-10 diagnosis statistics from the patient administrative system and population data from Statistics Norway. All data was collected per age groups and seasons, and from our study we calculated the number of children staying ≥24 hours with the detection of HMPV and RSV with LRTI diagnosis. Six children had both HMPV and RSV, and were included in the HMPV group. These ICD-10 codes were included: pneumonia J10.0. J11.0, J12.0-J12.9, J13-J15, bronchitis J20, bronchiolitis J21, unspecified LRTI J22 and asthma exacerbation J45-46. Hospitalization rates from the 2006/07 and 2014/15 seasons were not calculated, because the 2006/07 season had a shorter duration, and figures of RSV-specific LRTI diagnoses were missing from the spring of 2015.

The duration of HMPV shedding was estimated by Kaplan-Meier analysis in 32 children. In total, 93 respiratory specimens, in average 3 per child, were collected at a median 4.0, 8.5 and 13.0 days after symptom onset. Four HMPV-positive specimens in the last sampling were censored. Samples with Ct values >42 were encoded with a Ct value ≥42.1 for the HMPV shedding analysis.

We used the χ^2-test or Fischer's Exact Test, Student t-test, Mann-Whitney U-test or Kruskal-Wallis test to compare categorical, parametric and non-parametric variables, as

149 appropriate. Repeated measures were analyzed by Friedman test for ordinal variables and
150 Cochran's Q test for dichotomous variables. *P*-values < 0.05 (two-sided) were considered
151 statistically significant and the data was analyzed using IBM SPSS Statistics 22 and
152 SigmaPlot 13.0.

RESULTS

HMPV and RSV among children with RTI and asymptomatic controls

Among children with RTI, HMPV was detected in 7.3% (267/3,650), RSV in 28.7% (1048/3,650) and 64.0% had other viruses or were virus-negative (Figure 1A and Supplementary Table 1). Infected children with HMPV and RSV had a median age of 17.7 months (IQR 9.1-29.7) and 7.4 months (IQR 2.5-17.7) ($P < 0.001$), respectively. Three children were hospitalized twice with HMPV infection within a 5-year period, elicited by unknown or different subtypes. Among the asymptomatic controls with a median age of 39.4 months (IQR 21.0-63.3), HMPV was detected in 2.1% (7/339) and RSV in 3.2% (11/339) (Figure 1B). HMPV and RSV more frequently were detected among children with RTI than among controls (both $P < 0.001$). The median Ct-value of HMPV among children with RTI (28.0, IQR 24.2-32.1) was lower than among controls (38.9 (IQR 37.6-39.2, $P < 0.001$). In all 43.9% of infected children were HMPV culture-positive at admittance compared to none of the controls. Similarly, the median Ct-value of RSV among children with RTI (23.5, IQR 20.9-26.8) was lower than among controls (30.9, IQR 30.3-33.2, $P < 0.001$), and 91.4% (958/1048) and 54.5% (6/11) respectively, were RSV culture-positive in the same two groups.

Seasonal trends and epidemics

The detection of HMPV varied from 2.6% to 12.4% of the children in each of 9 seasons, an average of 7.3% per season (Figure 2 and Supplementary Table 1). RSV was more frequent than HMPV, and varied from 21.3% to 39.0%, an average of 28.7% per season. Analyses of the monthly HMPV-distribution during all nine years showed that HMPV mostly appeared from January to April (74.2%, 198/267). Going more into detail, HMPV appeared from January-March in 62.5%, April-June in 23.2%, October-December in 13.1% and July-September in 1.1%. Furthermore, the occurrence of HMPV in the period from January to

March in odd and even years (even year, i.e. 2006/07) was equal ($P = 0.730$) (Supplementary Figure 1). RSV was particularly frequent from January to March (71.2%, 746/1,048). Looking on epidemics, HMPV appeared from October to July in 2 to 6 consecutive months, with a median outbreak duration of 3.5 months (Figure 2 and Supplementary Table 1). Four seasons had peak activity in January and February, while the other four seasons had peak activity in March or later. The winter HMPV epidemics had higher peaks (winter: 11-20 HMPV-positives per month vs spring-summer: 3-8 HMPV-positives per month) and a longer duration (winter: median 5 months vs spring-summer: 2.5 months) than the spring-summer HMPV epidemics ($P = 0.004$ and $P = 0.057$, respectively). RSV-epidemics occurred in all 9 seasons and had a median duration of 5 months, varying from 5 to 8 months from October to July. RSV epidemics had a longer median duration than HMPV epidemics ($P = 0.011$). Additionally, HMPV epidemics appeared before, during or after RSV epidemics.

HMPV genotypes and subtypes

Genotype B was detected in 56.8% (126/222) and genotype A in 43.2% (96/222). HMPV A and B co-circulated each season, although the distributions of each genotype changed during the seasons ($P < 0.001$) (Figure 3). Among the HMPV genotype B positive samples, 37 were subtype B1 and 89 were subtype B2. In genotype A, 12 samples were subtype A2a, 80 were subtype A2b and 4 were subtype A2 (unassigned), while no samples were positive for subtype A1. Two or more subtypes were detected every season, and one or two subtypes dominated in each season. Phylogenetic analyses of the F-gene region showed that several strains circulated each year. No clusters or new strains were detected during the 9 year-long study period (see supplementary Figure 2).

201 **Hospitalizations rates of LRTI during 7 seasons**

202 Altogether, 900 children were hospitalized with LRTI with either HMPV (n = 146) or RSV (n
203 = 754). The mean annual hospitalization rate of HMPV-associated LRTI in children <5 years
204 was 1.8/1,000 children (Table 1). The youngest children aged 0-11 months old had a rate of
205 2.8/1,000 children, and 12-23 months old had a rate of 3.6/1,000 children. Children with RSV
206 had higher hospitalization rates than HMPV: 9.9/1,000 children <5 years, 27.0/1,000 children
207 aged 0-11 months and 12.9/1,000 children aged 12-23 months. In children ≥24 months, the
208 rates gradually decreased in both HMPV- and RSV-infected children with increasing age.

209 **Shedding of HMPV**

210 A Kaplan-Meier analysis estimated that 50% (median) and 100% of 32 children were virus-
211 PCR-negative after 13.0 (95% CI 11.5-14.5) and 28.0 days, respectively, from the onset of
212 symptoms (Figure 4), with the shedding time varying from 6.0-28.0 days. The NPA taken at
213 admittance had a median Ct value of 23.8 and 84.4% (27/32) were culture-positive. The first
214 follow-up samples had a median Ct value of 34.7, and only 15.6% (5/32) were still culture-
215 positive (Supplementary Table 2). The second follow-up samples had a median Ct value
216 ≥42.1, the value encoded for virus-negatives, and none out of 20 samples were culture-
217 positive. The median Ct values gradually increased, and the rate of culture-positive samples
218 gradually decreased from admittance to first and second follow-up samples (both $P < 0.001$),
219 and all children gradually improved.

220

DISCUSSION

The present data from our population-based study performed during nearly 9 years show that HMPV is associated with a substantial disease burden, and annually causes an average of 1.8 hospitalizations per 1,000 Norwegian children younger than 5 years, although HMPV is still associated with a five times lower hospitalization rate than RSV. Several findings have confirmed that HMPV is an epidemic virus: First, HMPV occurred in regular winter and spring-summer outbreaks during the entire study period. Secondly, the infected children initially had high viral levels, but a short viral shedding time, and thirdly, no asymptomatic controls had a HMPV-positive culture, although a few had low levels of HMPV as detected by PCR.

On average, HMPV was detected in 7.3% of all children admitted with RTI during the whole period, but it varied considerably from only 2.6% to 12.4% per season. Most previous studies from countries in the Northern hemisphere measured the occurrence over shorter periods, but found relative similar figures and seasonal variations [1, 3, 8, 13-15]. HMPV appeared mostly from January to April and regularly caused outbreaks of a median of 5 months' duration, peaking in the winter months. Smaller outbreaks with a median duration of 2.5 months appeared during the spring and early summer months, and coincided with a reduction in the total number of children admitted with RTI. In addition, the occurrence of HMPV was quite similar in both odd and even years, in contrast to observations from southern Europe, with alternating epidemics in winter and spring-summer every other year [15, 34]. We speculate as to whether this may be related to the cold climate in our country compared to the warmer climate in the southern part of Europe [35]. RSV outbreaks occurred in every season and lasted an average of 5 months, and most often peaked in January to March. As previously described, HMPV outbreaks appeared before, overlapping with or after RSV [5].

246 We detected all known HMPV subtypes, except for subtype A1, with subtype B2 being the
247 most frequent over the entire period. In line with other studies [6, 7, 15, 19], the distribution
248 of subtypes showed great seasonal variation. In every season one or two subtypes dominated,
249 and at least two subtypes circulated, but no new strains or clusters were detected. We
250 previously have reported that HMPV genotypes and subtypes were associated with very
251 similar clinical manifestations [4].

252 In the present study, the average annual hospitalization rate of HMPV-related LRTI
253 over 7 seasons was 1.8/1,000 children aged <5 years old. Children in the youngest age groups
254 had higher rates. We used a strict definition of severe HMPV infection including only
255 children with a hospital stay ≥24 hours and LRTI, which might explain why our estimates
256 differ from three US studies that included a broader spectrum of respiratory infections, and
257 reported estimates from 1.0- to 1.2/1,000 children <5 years old [1, 20, 21]. Two European
258 studies reported HMPV-related hospitalizations rates comparable with ours. A study from
259 Spain [23], based on 3 seasons, reported that 2.6/1,000 children <3 years old were
260 hospitalized, and in a single season study from UK [22] the rate was reported to be 1.3/1,000
261 children <6 years old. Our finding of higher hospitalization rate in 12-23 months-old children
262 differ with the findings in all previous studies [1, 20-23], and may also relate to our strict
263 inclusion criteria. The hospitalization rates of children with RSV-related LRTI in our study
264 were in line with findings from previous Norwegian [36], European [37, 38] and American
265 studies [39, 40], thereby confirming that HMPV causes hospitalization less often than RSV in
266 Europe and US.

267 To test the hypothesis that low detection rates and low levels of HMPV in healthy
268 children may be a result of virus shedding after previous RTI, we first measured the rate of
269 HMPV-positive samples among a group of asymptomatic children. A few percent had a
270 positive PCR test with high Ct levels, thus corresponding to low viral loads, but all were

virus-negative by culture. We also studied a group of children with HMPV infection with repeated specimens sampled, who had low Ct values (high viral loads) and a high rate of positive cultures initially. During the progress of the disease, these children improved clinically, viral loads gradually decreased and all became virus-negative by culture after 13 days. Despite these changes, half of the children were still virus-positive by PCR test after 13 days and all were negative after 28 days only. Taken together, our observations along with observations done by others [1, 30, 31, 41, 42], support that a positive PCR test for HMPV in healthy children is unlikely to indicate an asymptomatic infection, and we speculate whether it instead indicates the presence of small amounts of viral nucleic acids after a previous HMPV infection. Others [33, 41] have demonstrated a 2-3-week-long shedding time in children with RSV infection, which in a similar way may explain the low detection rate of RSV at low viral levels in the controls of the present study.

As indicated by the hospitalization rates, the incidence of severe HMPV infection, decreased by age. In addition, only 1% of previously healthy children were admitted with recurrent HMPV infections elicited by unknown or different HMPV subtypes. Previous research has shown that most children become seropositive during the first 5 years of life [43], while data from experimental studies suggest that certain HMPV subtypes may not stimulate an adequate immune response in all cell types [44]. However, our clinical data support that healthy children usually develop a robust immunity against most HMPV subtypes during childhood. On the other hand, outside a hospital setting, others have shown that HMPV may still cause recurrent mild RTI in children [45] and adults [46]. Moreover, children [47] and adult [48] with impaired immunity may be prone to severe HMPV infections, even with a high seroprevalence at all ages [49].

It is a strength of the present population-based study, that we prospectively enrolled children at all ages from the same county in mid-Norway, and to the only existing pediatric

hospital in this region during a long period. It is also an advantage that we used the same PCR tests and viral cultivation methods during the entire period. However, the controls were sampled during anesthesia and we have not adjusted for the fact that controls were in general older than children with RTI. Moreover, controls were not contacted after sampling to assess whether subsequent RTI symptoms had occurred. All factors might have contributed to higher viral detection rates among controls. Some HMPV-positive samples were not genotyped and a few were unassigned A2. Hence, the A1 subtype might have been present, and the pattern of circulating HMPV subtypes might have been even more heterogenic than described.

In conclusion, HMPV occurs in winter and spring-summer epidemics in Norwegian children, but the hospitalization rate is 5 times lower than RSV. All known HMPV subtypes, except for A1, circulate in Norway. Children are rarely hospitalized twice with HMPV infection. Children has a short HMPV shedding time and may not be infectious for more than 13 days, and the short shedding time may also explain the low HMPV detection rate among asymptomatic children.

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FIGURE LEGENDS

Figure 1. Study flow chart, where (A) represents children admitted with acute respiratory tract infections from November 2006 to July 2015 and (B) represents hospital controls admitted for elective surgery from June 2007 to April 2015.

HMPV indicates human metapneumovirus and RSV indicates respiratory syncytial virus.

Figure 2. Detection of HMPV and RSV among children with respiratory tract infection according to month and season.

Gray shade represents the total number of samples tested, with numbers at the right Y-axis. Dashed lines indicate the detection of HMPV (red) and RSV (blue) and solid lines indicates HMPV epidemic (≥10% positive) (red) and RSV epidemic (≥10% positive) (blue), with numbers at the left Y-axis. Diamonds are peak activity month during HMPV epidemics (red) and RSV epidemics (blue). During the 2007/08 season, no HMPV epidemic occurred and therefore no peak activity month is marked. The peak activity month during an epidemic was the month with highest number of children with the respective virus.

HMPV indicates human metapneumovirus and RSV indicates respiratory syncytial virus.

Figure 3. Distribution of human metapneumovirus (HMPV) subtypes, by season and month.

Number of children on the Y-axis and total HMPV (black solid line) indicates the total number of HMPV-positive samples, both genotyped and unknown genotype.

*Unassigned A2.

Figure 4. Kaplan-Meier analysis of shedding time of human metapneumovirus (HMPV) in respiratory samples of children with respiratory tract infection.

Y-axis represents estimated proportion of HMPV-positive samples and X-axis represents number of days from onset of symptoms until HMPV-negative sample. The estimated proportion (solid line) is presented with the 95% confidence interval (stippled lines).

Notes

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Potential conflicts of interests: All authors declare that they have no commercial or other associations that might pose a conflict of interests.

Presentations of data on meetings: The data has not been presented on international meetings yet.

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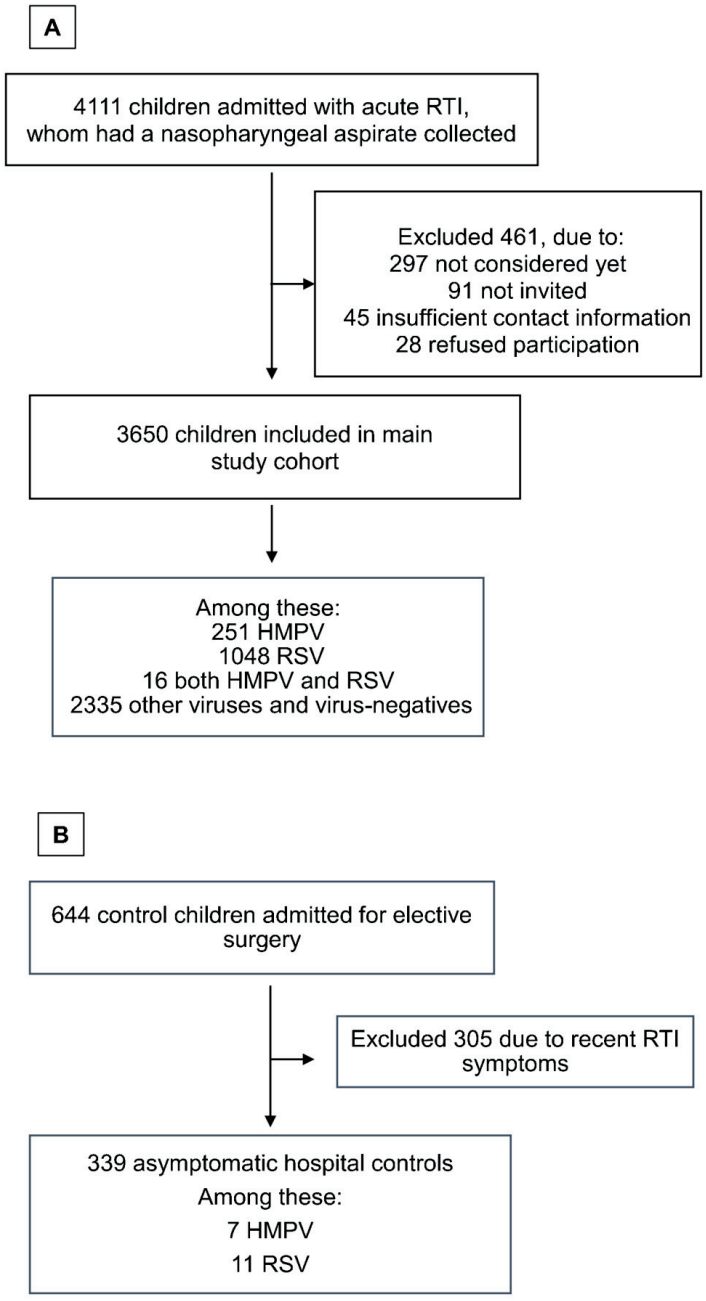

A
4111 children admitted with acute RTI, whom had a nasopharyngeal aspirate collected
Excluded 461, due to:
297 not considered yet
91 not invited
45 insufficient contact information
28 refused participation
3650 children included in main study cohort
Among these:
251 HMPV
1048 RSV
16 both HMPV and RSV
2335 other viruses and virus-negatives
B
644 control children admitted for elective surgery
Excluded 305 due to recent RTI symptoms
339 asymptomatic hospital controls
Among these:
7 HMPV
11 RSV

Figure 1

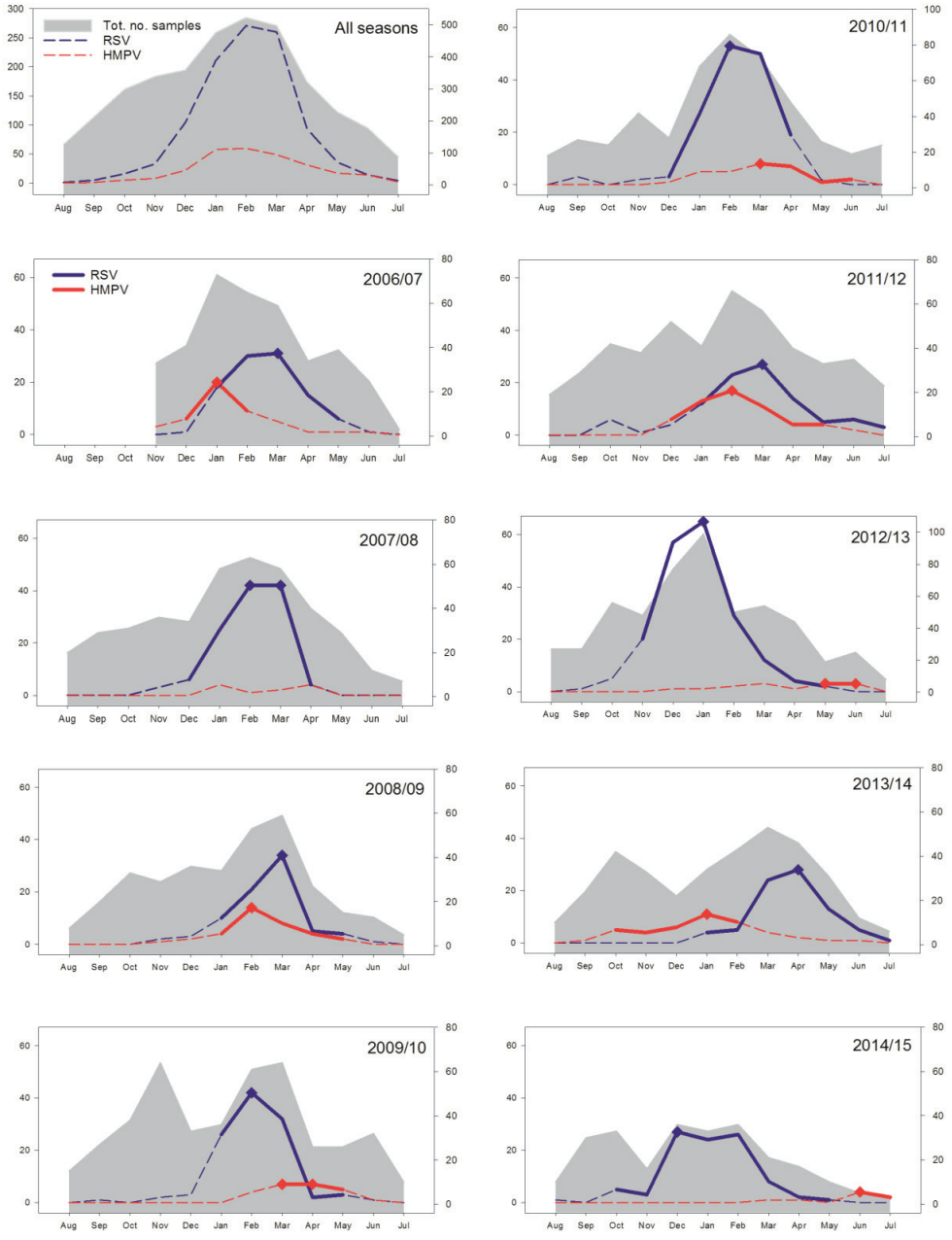

Tot. no. samples
RSV
HMPV
All seasons
2010/11
2006/07
2011/12
2007/08
2012/13
2008/09
2013/14
2009/10
2014/15
Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul

Figure 2

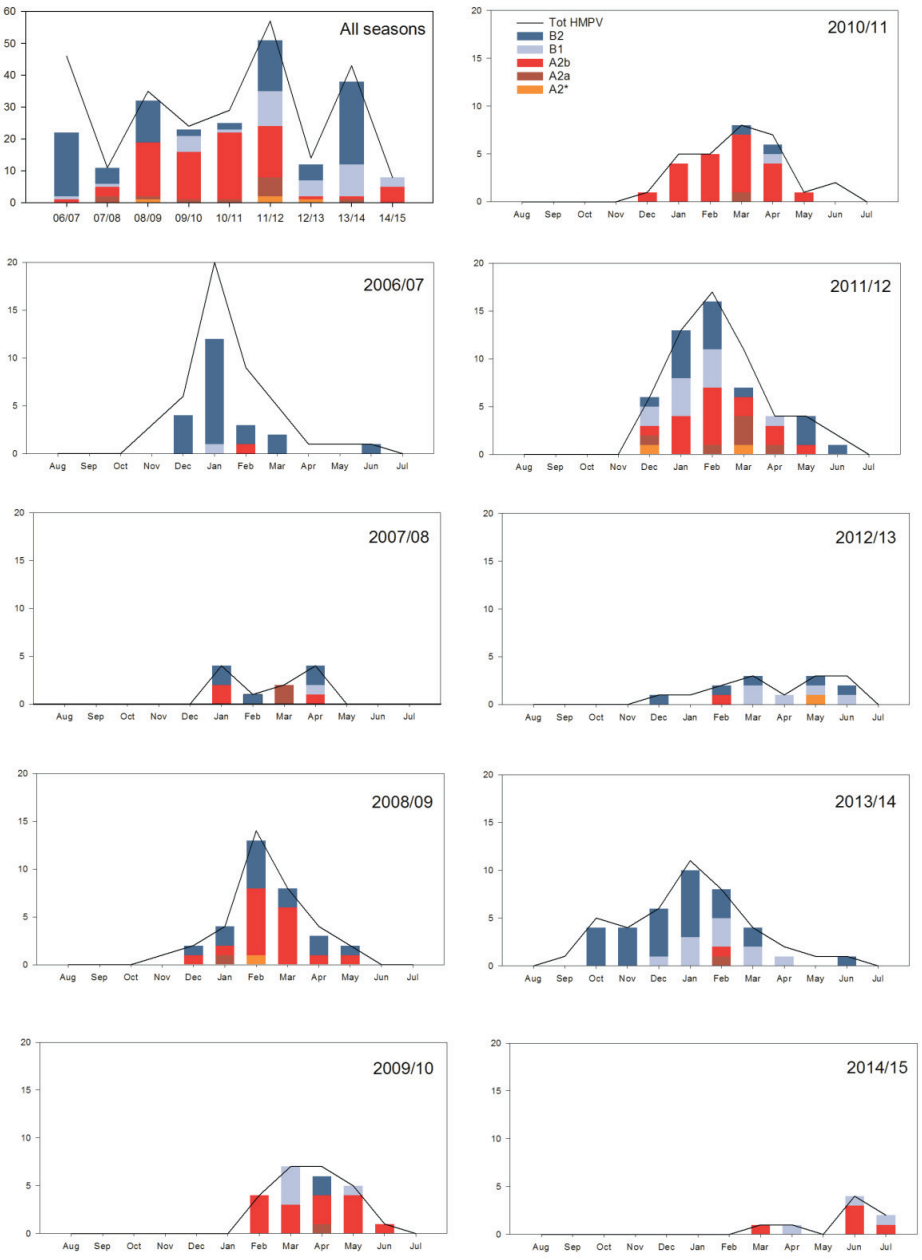

All seasons
06/07
07/08
08/09
09/10
10/11
11/12
12/13
13/14
14/15
Tot HMPV
B2
B1
A2b
A2a
A2*
2010/11
2006/07
2011/12
2007/08
2012/13
2008/09
2013/14
2009/10
2014/15
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul

Figure 3

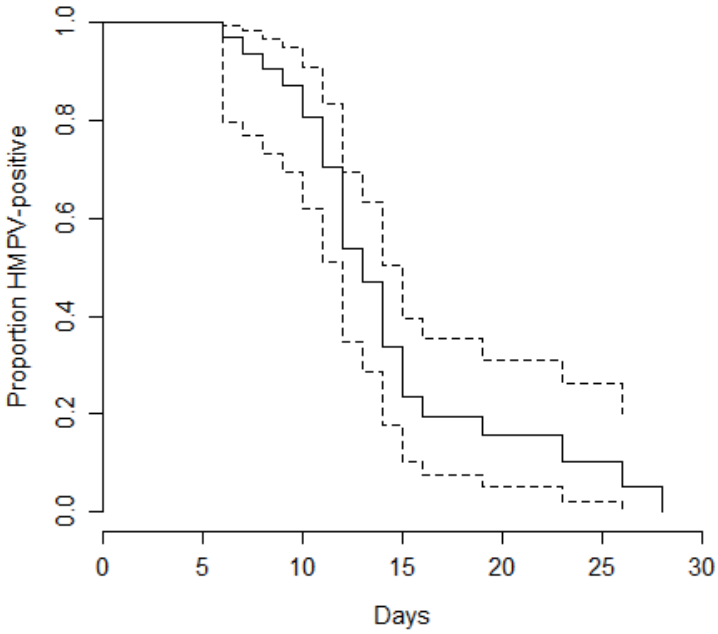
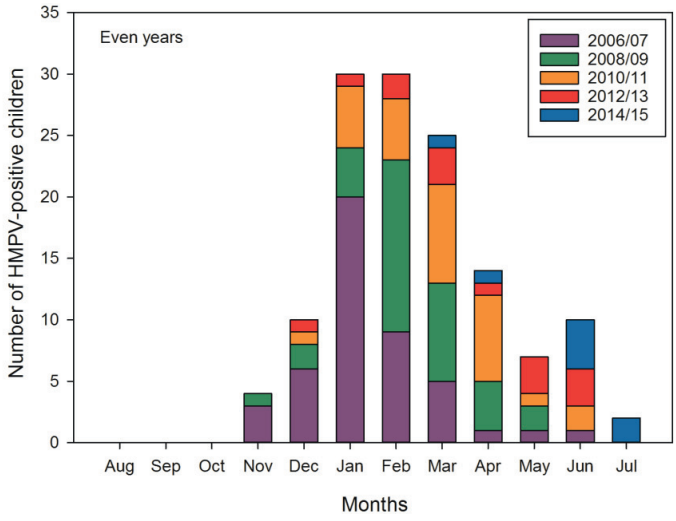

Proportion HMPV-positive
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
25
30
Days

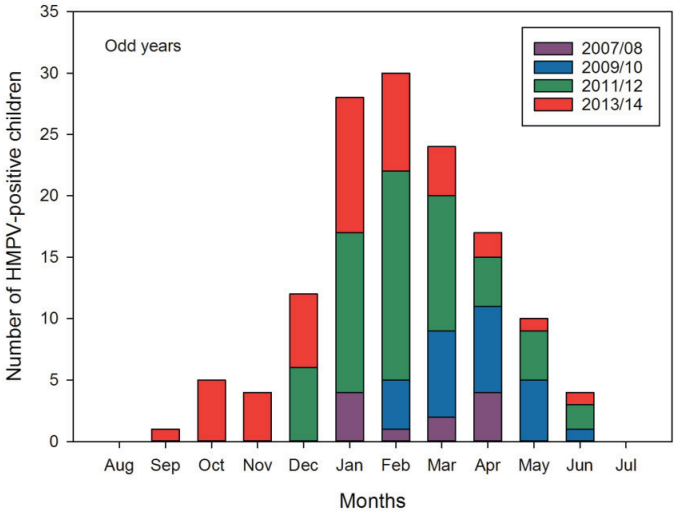
Figure 4

Table 1. Incidence Rates of Hospitalization per 1000 children with Lower Respiratory Tract Infection, by Virus (HMPV or RSV), Season and Age

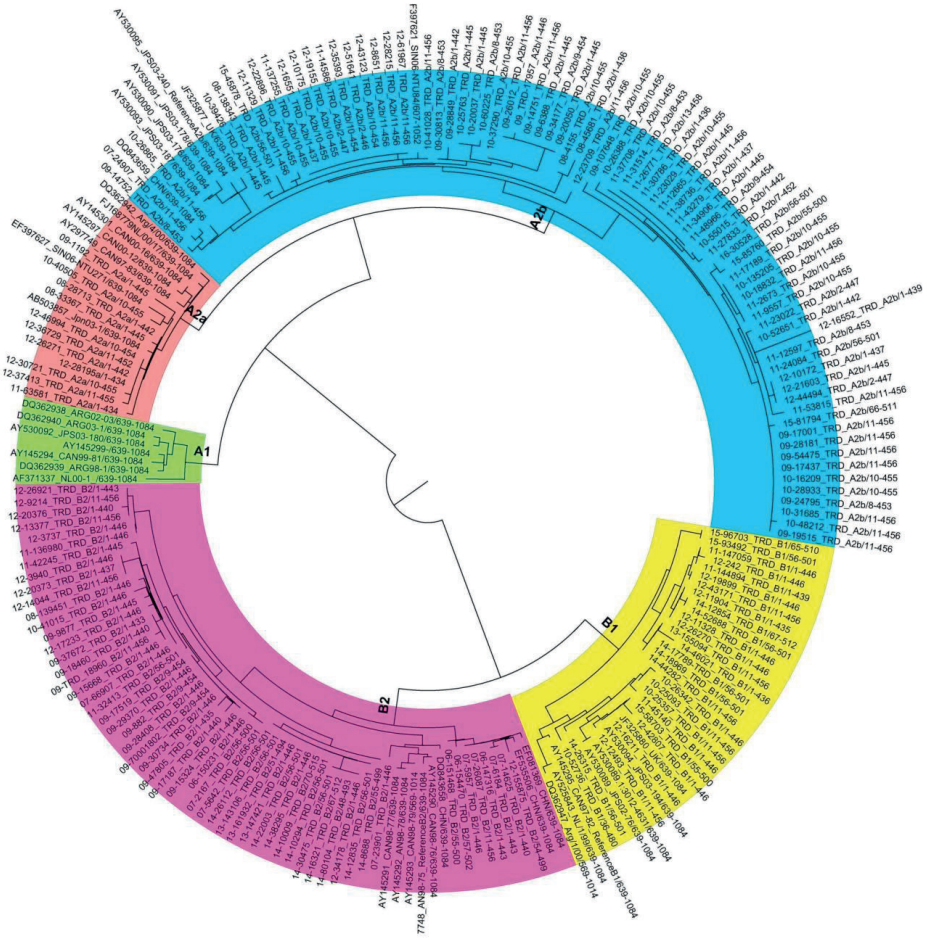
	Age									
	0-11 months		12-23 months		24-59 months		5-16 years		0-59 months	
Season	HMPV	RSV	HMPV	RSV	HMPV	RSV	HMPV	RSV	HMPV	RSV
2007/08	0.5	35.2	2.4	8.9	0.0	3.3	0.0	0.0	0.5	11.6
2008/09	4.0	19.7	5.0	13.4	1.2	1.5	0.1	0.1	2.5	8.3
2009/10	3.4	25.2	1.0	13.6	1.6	2.5	0.0	0.0	1.6	9.5
2010/11	2.4	31.8	2.5	12.9	0.6	3.7	0.0	0.1	1.3	12.1
2011/12	5.2	18.2	6.9	12.6	2.1	1.3	0.1	0.0	3.7	7.3
2012/13	1.5	40.7	1.3	19.4	0.5	2.9	0.0	0.1	0.8	14.1
2013/14	2.7	18.2	6.4	10.1	1.2	1.5	0.1	0.0	2.4	6.6
Mean	2.8	27.0	3.6	12.9	1.0	2.4	0.04	0.04	1.8	9.9
SD	1.6	9.0	2.4	3.3	0.7	1.0	0.05	0.05	1.1	2.8

Abbreviations: HMPV, Human Metapneumovirus; RSV, Respiratory Syncytial virus; SD, Standard Deviation.





Supplementary Figure 1. Occurrence of human metapneumovirus (HMPV) in even and odd years.
Even years, i.e. 2006/07.



Supplementary Figure 2. Phylogeny of 169 patient sequencesobtained by partial sequencing of the hMPV F-gene and 36 GenBanksequences. Phylogenetic analysis was constructed by the Neighbour-Joining method with evolutionary distances calculated by the Tamura-Nei method using the Geneious v.9.0.2 software. The sequences from this study are labelled by year of sample collection,specimen identifier and TRD (Trondheim). The GenBank strains are labelled with accession number and geographic origin. ARG,Argentina; AUS, Australia; CAN, Canada; CHN, China;J PS, Japan; NL, Netherlands; SIN, Singapore; UK, United Kingdom. The figure is produced using the FigTree v.1.4.3 program.

Supplementary Table 1. Seasonal and Monthly detection of Human Metapneumovirus (HMPV) and Respiratory Syncytial virus (RSV) among 3650 children with Respiratory Tract Infections

Season	NPA[a] result	Month												Total
		Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	
06/07	HMPV				3	**6**	**20**	**9**	5	1	1	1	0	46
	RSV				0	1	**18**	**30**	**31**	**15**	**6**	1	0	102
	Other[b]				30	34	35	26	23	18	32	23	3	224
	Total				33	41	73	65	59	34	39	25	3	372
07/08	HMPV	0	0	0	0	0	4	1	2	4	0	0	0	11
	RSV	0	0	0	3	**6**	**25**	**42**	**42**	**4**	0	0	0	122
	Other	20	29	31	33	28	29	20	14	32	29	12	7	284
	Total	20	29	31	36	34	58	63	58	40	29	12	7	417
08/09	HMPV	0	0	0	1	2	**4**	**14**	**8**	**4**	**2**	0	0	35
	RSV	0	0	0	2	3	**10**	**21**	**34**	**5**	**4**	1	0	80
	Other	8	20	33	26	31	20	18	17	18	9	12	5	217
	Total	8	20	33	29	36	34	53	59	27	15	13	5	332
09/10	HMPV	0	0	0	0	0	0	4	**7**	**7**	**5**	1	0	24
	RSV	0	1	0	2	3	**26**	**42**	**32**	**2**	**3**	1	0	112
	Other	15	26	38	62	30	10	15	25	17	18	30	10	296
	Total	15	27	38	64	33	36	61	64	26	26	32	10	432
10/11	HMPV	0	0	0	0	1	5	5	**8**	**7**	**1**	**2**	0	29
	RSV	0	3	0	2	**3**	**27**	**53**	**50**	**19**	2	0	0	159
	Other	18	24	24	40	24	36	28	15	22	23	17	24	295
	Total	18	27	24	42	28	68	86	73	48	26	19	24	483
11/12	HMPV	0	0	0	0	**6**	**13**	**17**	**11**	**4**	**4**	2	0	57
	RSV	0	0	6	1	4	**12**	**23**	**27**	**14**	**5**	**6**	**3**	101
	Other	19	29	36	37	42	16	26	19	22	24	27	20	317
	Total	19	29	42	38	52	41	66	57	40	33	35	23	475

12/13	HMPV	0	0	0	0	1	1	2	3	1	**3**	**3**	0	14
	RSV	0	1	5	**20**	**57**	**65**	**29**	**12**	**4**	**2**	0	0	195
	Other	27	26	51	28	19	33	19	39	39	14	22	8	325
	Total	27	27	56	48	77	99	50	54	44	19	25	8	534
13/14	HMPV	0	1	**5**	**4**	**6**	**11**	**8**	4	2	1	1	0	43
	RSV	0	0	0	0	0	**4**	**5**	**24**	**28**	**13**	**5**	**1**	80
	Other	10	23	37	29	16	19	30	25	16	17	6	5	233
	Total	10	24	42	33	22	34	43	53	46	31	12	6	356
14/15	HMPV	0	0	0	0	0	0	0	1	1	0	**4**	**2**	8
	RSV	1	0	**5**	**3**	**27**	**24**	**26**	**8**	**2**	**1**	0	0	97
	Other	9	30	28	13	9	9	10	12	14	9	1	0	144
	Total	10	30	33	16	36	33	36	21	17	10	5	2	249
Total	HMPV	0	1	5	8	22	58	60	49	31	17	14	2	267
all	RSV	1	5	16	33	104	211	271	260	93	36	14	4	1048
seasons	Other	126	207	278	298	233	207	192	189	198	175	150	82	2335
	Total	127	213	299	339	359	476	523	498	322	228	178	88	3650

[a]Nasopharyngeal aspirates.

[b]Virus-negatives and other viruses than HMPV and RSV.

Bold figures represent epidemics of HMPV and RSV. Epidemics defined as time between onset month and offset month during one season. The onset month was the first of two consecutive months when the monthly proportion of each virus ≥ 10% positive and offset month was the last month when the monthly proportion of each virus ≥ 10% positive, preceding 2 consecutive months with monthly proportion of each virus < 10% positive, out of the total number of NPA each month during same season.

Supplementary Table 2. HMPV viral loads and HMPV culture results at three sampling times and days with symptoms in children with respiratory tract infections

Child no.	Sampling at admittance			First follow-up sampling			Second follow-up sampling		
	Days[a]	Ct-value[b]	Viral Culture[c]	Days	Ct-value	Viral Culture	Days	Ct-value†	Viral Culture
1	2	19.2	Pos.	4	23.6	Neg.	11	≥42.1	Neg.
2	6	22.2	Pos.	12	≥42.1	Neg.			
3	7	29.1	Pos.	9	38.6	Neg.	14	≥42.1	Neg.
4	5	33.1	Neg.	9	≥42.1	Neg.			
5	6	28.8	Pos.	7	29.5	Neg.	11	≥42.1	Neg.
6	3	23.9	Pos.	6	22.7	Pos.	10	37.9	Neg.
7	4	24.9	Pos.	6	27.4	Neg.			
8	5	21.3	Pos.	10	33.9	Neg.	16	≥42.1	Neg.
9	2	21.4	Pos.	6	31.1	Neg.	11	≥42.1	Neg.
10	6	30.5	Pos.	10	33.3	Neg.	15	≥42.1	Neg.
11	3	25.1	Pos.	10	≥42.1	Neg.			
12	2	23.6	Pos.	7	29.9	Pos.	10	32.0	Neg.
13	2	22.9	Pos.	6	34.2	Pos.	12	≥42.1	Neg.
14	5	31.5	Neg.	12	≥42.1	Neg.			
15	6	20.5	Pos.	17	27.9	Neg.	22	30.4	Neg.
16	2	21.7	Pos.	4	25.5	Pos.	6	30.8	Neg.
17	3	28.8	Neg.	7	32.4	Neg.	10	38.0	Neg.
18	4	27.2	Pos.	12	≥42.1	Neg.			
19	4	27.4	Pos.	8	≥42.1	Neg.			
20	3	19.3	Pos.	7	36.2	Neg.	11	36.7	Neg.
21	5	33.7	Neg.	9	38.2	Neg.	14	≥42.1	Neg.

22	2	20.7	Pos.	7	31.9	Neg.	14	≥42.1	Neg.
23	4	30.9	Neg.	6	≥42.1	Neg.			
24	5	22.2	Pos.	12	30.7	Neg.	17	37.4	Neg.
25	5	24.5	Pos.	10	35.2	Neg.	15	≥42.1	Neg.
26	2	23.3	Pos.	6	26.2	Neg.	9	35.4	Neg.
27	5	28.3	Pos.	10	≥42.1	Neg.			
28	4	21.2	Pos.	13	≥42.1	Neg.			
29	5	20.2	Pos.	8	29.0	Pos.	15	≥42.1	Neg.
30	5	21.5	Pos.	14	36.6	Neg.	20	33.8	Neg.
31	5	26.1	Pos.	19	≥42.1	Neg.			
32	2	23.8	Pos.	7	≥42.1	Neg.			
Median[d]	4.0	23.8		8.5	34.7		13.0	≥42.1	
Positive cultures, n (%)			27 (84.4)			5 (15.6)			0 (0.0)

Abbreviations; Ct-value, cycle-threshold value; HMPV, human metapneumovirus.

[a]Days with symptoms from onset of respiratory tract infection to sampling at admittance, first and second follow-up samples.

[b]Ct-value in respiratory samples from sampling at admittance, first and second follow-up samples.

[c]Viral cultures positive or negative for HMPV.

[d]Median days with symptoms and Ct-values from sampling at admittance, first and second follow-up samples, where virus negatives were encoded with a Ct-value of ≥42.1.

Paper II

PLOS ONE

RESEARCH ARTICLE

Comparing Human Metapneumovirus and Respiratory Syncytial Virus: Viral Co-Detections, Genotypes and Risk Factors for Severe Disease

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Abstract

Background

It is unclarified as to whether viral co-detection and human metapneumovirus (HMPV) genotypes relate to clinical manifestations in children with HMPV and lower respiratory tract infection (LRTI), and if the clinical course and risk factors for severe LRTI differ between HMPV and respiratory syncytial virus (RSV).

Methods

We prospectively enrolled hospitalized children aged <16 years with LRTI from 2006 to 2015. Children were clinically examined, and nasopharyngeal aspirates were analyzed using semi-quantitative, real-time polymerase chain reaction tests for HMPV, RSV and 17 other pathogens. HMPV-positive samples were genotyped.

Results

A total of 171 children had HMPV infection. HMPV-infected children with single virus (n = 106) and co-detections (n = 65) had similar clinical manifestations. No clinical differences were found between HMPV genotypes A (n = 67) and B (n = 80). The HMPV-infected children were older (median 17.2 months) than RSV-infected children (median 7.3 months, n = 859). Among single virus-infected children, no differences in age-adjusted LRTI diagnoses were found between HMPV and RSV. Age was an important factor for disease severity among single virus-infected children, where children <6 months old with HMPV had a milder disease than those with RSV, while in children 12–23 months old, the pattern was the opposite. In multivariable logistic regression analysis for each virus type, age ≥12 months (HMPV), and age <6 months (RSV), prematurity, ≥1 chronic disease and high viral loads of RSV, but not high HMPV viral loads, were risk factors for severe disease.

collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

Among hospitalized children with LRTI, HMPV manifests independently of viral co-detections and HMPV genotypes. Disease severity in HMPV- and RSV-infected children varies in relation to age. A history of prematurity and chronic disease increases the risk of severe LRTI among HMPV- and RSV-infected children.

Introduction

Since the discovery of human metapneumovirus (HMPV) in 2001 [1], studies from all parts of the world have shown that HMPV causes respiratory tract infection (RTI) in children [2–7]. HMPV is usually detected in airway secretions from children with RTI by use of polymerase chain reaction (PCR) tests, and the virus seldom appears in healthy children [8]. HMPV has been classified into the *Pneumoviridae* family. Two main genotypes (A and B) and at least four genetic subtypes (A1, A2, B1 and B2) exist [9–12]. Whether these genotypes cause similar or different infections is largely unclarified because some studies have shown quite similar manifestations [13–15], whereas others found that either genotypes A [16,17] or B [18] may cause more severe disease.

Using sensitive nucleic-acid based molecular tests to diagnose viral pathogens has revealed that many children with LRTI have more than one virus present in the respiratory tract [14,19,20]. Previous studies in HMPV-infected children found that such viral co-detections were associated with increased disease severity [21,22]. However, this was not confirmed in other studies [14,17,19,23].

HMPV is closely related to respiratory syncytial virus (RSV), by far the most important airway virus affecting children worldwide [6,14,20,24]. It has been reported that RSV and HMPV infections in children may be quite similar [23,25,26], but there is also some evidence that RSV causes more severe disease than HMPV [6,18]. Risk factors for severe RSV infection are young age, prematurity, chronic lung disease, chronic heart disease and severe neurological disabilities [18,24,27–30]. Although it has not been studied to the same extent, it seems that some of these factors may also increase the risk of severe HMPV infection [2,17,18,29,31–33]. With some exceptions [18,29], most studies have separately dealt with risk factors for severe HMPV and RSV infections.

In the present study, we prospectively enrolled a large cohort of children <16 years old, who were admitted to hospital with acute RTI during a nearly 9-year long period from 2006 to 2015, and diagnosed a broad panel of respiratory viruses. Our primary aim was to study clinical manifestations in children with HMPV lower respiratory tract infection (LRTI), taking viral co-detections and HMPV genotypes into account. Our secondary aim was to compare HMPV- and RSV-infected children with LRTI, with a special emphasis on clinical manifestations and risk factors for severe disease.

Materials and Methods

Study Design and Population

During the period from November 2006 to August 2015, we prospectively enrolled children aged <16 years who were admitted with acute RTI at the Pediatric Emergency Department, at the Department of Pediatrics, St. Olav's Hospital, University Hospital of Trondheim, Norway, and who were sampled on clinical indications with a nasopharyngeal aspirate (Fig 1). The

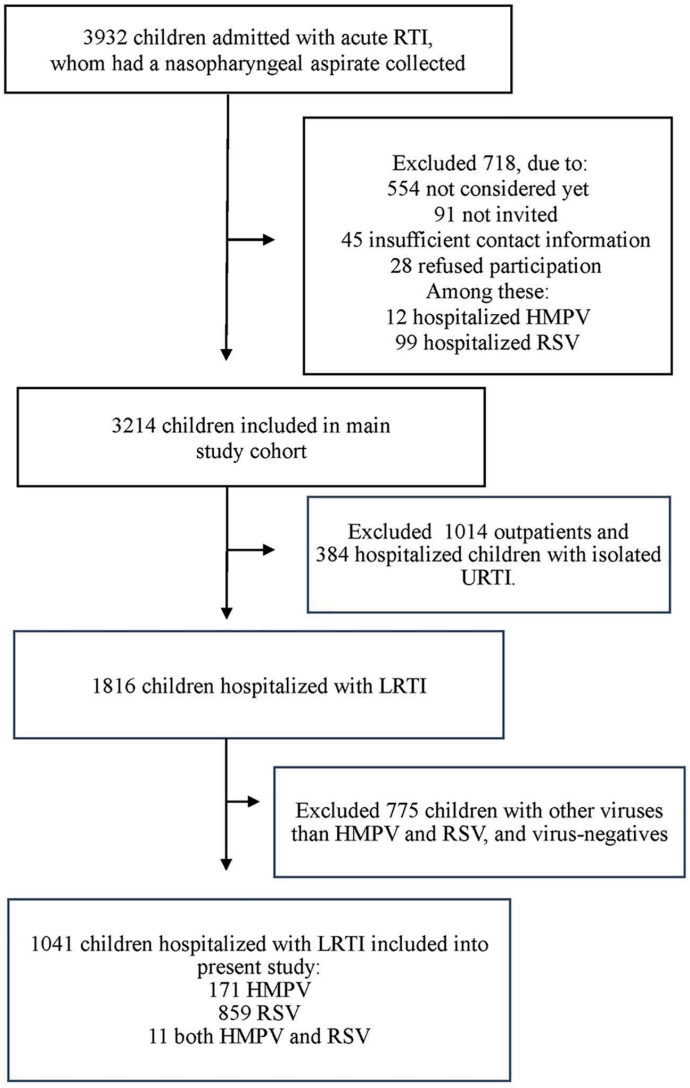

Fig 1. Study flow chart. Children were enrolled from the beginning of November 2006 to the end of July 2015. HMPV, human metapneumovirus; LRTI, lower respiratory tract infection; RSV, respiratory syncytial virus; RTI, respiratory tract infection; URTI, upper respiratory tract infection.

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hospital provides care for 58,000 children younger than 16 years and 18,000 children younger than five years of age from Sør-Trøndelag County in Mid-Norway (Statistics Norway). Written informed consent to participate was collected from children ≥12 years and caregivers to most of the children during the hospital stay. However, due to practical challenges, including dealing with children 24 hours a day, we also enrolled some children after hospital discharge. Their caregivers received written information per regular mail, and the child was included if

the caregivers did not resist enrollment by contacting the hospital within two weeks. The latter inclusion method was also approved by the Regional Committee for Medical and Health Research Ethics. Some children were included more than one time if they were hospitalized with distinct RTI episodes, whereas recurrent hospitalizations due to the same RTI were only registered once. Exclusion criteria were children evaluated for RTI who were hospitalized mainly due to other diseases, such as newborns not dismissed from the hospital, children with cytostatic or immune-suppressive treatment, and children with other infections such as gastroenteritis and urinary tract infection. We systematically collected baseline characteristics and past and current medical history from caregivers, who filled out a questionnaire, while clinical information was abstracted from medical records. Characteristics included gestational age at birth, age, gender, siblings, day care attendance and chronic diseases. Preterm birth was defined as a gestational age <36 weeks of gestation, and age was categorized according to clinically relevant groups. Chronic airway diseases, other than asthma, included bronchopulmonary dysplasia, congenital airway malformations, cystic fibrosis and neuromuscular disorders with hypoventilation in need of respiratory support and daily inhalations for mucus clearance. Tachypnea was defined as a ≥ 10 increase in normal respiratory rate according to the child's age [34]. Several children had more than one chronic disease, i.e. both asthma and other chronic airway disease, or both cerebral palsy and epilepsy. Hence, for the logistic regression analyses, we used a combined binary variable: "≥ 1 chronic disease" (coded: no chronic disease or ≥ 1 chronic disease). Nasopharyngeal aspirates were collected within 24 hours of presentation in the vast majority of the children. Children with a stay <24 hours were categorized as outpatients, while children who stayed ≥ 24 hours and were admitted to the wards of the Pediatric Department, were categorized as inpatients. From the main cohort of children with acute RTI, we selectively included HMPV- and RSV-infected children hospitalized with LRTI into the present study (Fig 1). Consequently, outpatients, children admitted with isolated upper RTI (URTI) and children admitted with LRTI and viruses other than HMPV or RSV, and virus negatives, were excluded (Fig 1). The Regional Committee for Medical and Health Research Ethics approved the study.

Clinical Investigations and Diagnostic Criteria

All children were routinely treated at the discretion of the medical doctors at the Department of Pediatrics. Children with one or more characteristic manifestations of rhino-pharyngitis, tonsillitis, conjunctivitis, otitis media or acute laryngitis were diagnosed with URTI. LRTI was categorized into five categories based on clinical manifestations and radiological findings: 1) Bronchiolitis: age <24.0 months old, tachypnea or signs of lower airways obstruction and a normal radiogram or a radiogram with air trapping/hyperinflation, perihilar infiltrates and/or atelectasis, 2) Obstructive bronchitis: age ≥ 24.0 months old, signs of lower airway obstruction and a normal radiogram or a radiogram with air trapping/hyperinflation, perihilar infiltrates and/or atelectasis, 3) Pneumonia: presence of dyspnea with or without auscultatory findings such as muffled respiratory sounds and a radiogram with localized or lobular infiltrates, or pleural effusion, 4) Asthma exacerbation: signs of lower airway obstruction and either a current asthma diagnosis or two or more previous episodes with lower airway obstruction during the previous year, or one or more episodes of lower airway obstruction and atopic status (eczema, allergy), and 5) Unspecified LRTI: cough or other signs of LRTI without signs of lower airway obstruction, with or without auscultatory findings and a normal radiogram or radiogram with perihilar infiltrates and/or atelectasis. Some asthmatic children who developed symptoms and signs of pneumonia were categorized with pneumonia.

Respiratory Support and Disease Severity Measures

Respiratory support (RS) with a non-invasive ventilator (NIV) included positive airway pressure via mask in continuous or bi-level modes, or via a high-flow nasal cannula. Prior to 2011, all children in need of NIV were admitted to the Pediatric Intensive Care Unit (PICU). During 2011, respiratory support with high-flow nasal cannula was introduced at the wards, which reduced the need for PICU admissions. Invasive ventilator (IV) support was defined as RS applied by endotracheal tube or tracheostomy, and children with acute respiratory failure in need of IV were admitted to the PICU. Initiation of any RS, or an increase in baseline RS for those with chronic RS, were defined as RS related to the acute RTI. To adjust for our new practice performing NIV treatment at the wards, we defined a disease severity score reflecting disease severity independently of treatment location. This score, ranging from 0 to 4 points, was defined as the sum of: 1) a need for oxygen to maintain oxygen saturation $\geq$93% (1 point); 2) length of stay $\geq$6 days, corresponding to or above the 75 percentile limit for all hospitalized children with LRTI (1 point); 3) a need for respiratory support with NIV (1 point); 4) a need for respiratory support with both NIV and IV (2 points); and 5) need of IV support (2 points). In addition, admission to the PICU, was reported as a disease severity measure. Severe disease was defined as a disease severity score $\geq$2 points, corresponding to- or above the 75% percentile limit among all hospitalized children with LRTI.

Laboratory Investigations

Nasopharyngeal aspirates (NPA) were collected and placed into a standard virus transport medium without antibiotics, and analyzed at the Department of Medical Microbiology, St. Olavs Hospital. The detection of respiratory pathogens was done using in-house TaqMan real-time PCR assays, and semi-quantitative results were reported based on the cycle threshold value (Ct-value). A high viral load was defined as a Ct-value <28, a medium viral load with Ct-values of 28–35, and a low viral load with Ct-values >35. Ct-values above 42 were regarded as virus-negative. In-house real-time PCR panels included analysis for human adenovirus, human bocavirus, human coronavirus OC43, NL63, 229E, human enterovirus, human parechovirus, HMPV, influenza A virus, influenza B virus, parainfluenza virus types 1–4, RSV, human rhinovirus, *Bordetella pertussis*, *Chlamydophila pneumoniae* and *Mycoplasma pneumonia* [35]. Conventional viral cultures were also performed. The detection of HMPV as a single virus in the NPA is named single HMPV, while HMPV co-detected with $\geq$1 other virus is named HMPV with co-detection. Similar expressions are also used for RSV. The HMPV-positive specimens were genotyped by real-time PCR and DNA sequencing using primers targeting the F-gene of HMPV [11]. A 527-bp amplicon was sequenced using a Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequences were analyzed on an ABI 3130XL (Applied Biosystems). Genotypes were determined by comparing sequenced data with the nucleotide BLAST database (www.ncbi.nlm.nih.gov/BLAST/). Some of the NPA were not type-able due to a low viral load, and others were empty after regular PCR tests.

Blood samples were collected to measure the concentration of C-reactive protein (CRP) in mg/L and white blood cell count x 10^9/L.

Statistical Analyses

To compare categorical variables, we used the χ or Fisher exact tests when expected values were <5. Means of normally distributed continuous variables were compared by Student t-test or ANOVA, and non-normally distributed continuous or ordinal variables were compared by use of Mann-Whitney *U* or Kruskal-Wallis tests. We compared HMPV and RSV using

stratified analyses among those with single virus infections and those with viral co-detections. Children with both HMPV and RSV were excluded from these analyses. Due to significant age differences between HMPV- and RSV-infected children, we also used a stratification strategy to control for age. We analyzed risk factors for severe disease in HMPV- and RSV-infected children separately, using logistic regression. We dichotomized the combined disease severity score, defining cases as a score ≥ 2 and controls as a score <2, and used this as a binary outcome in the logistic regression analyses. We predefined factors related to exposure and outcome to be age, prematurity, ≥ 1 chronic disease, viral co-detection and Ct-values. All factors were entered into full multivariable logistic regression models. Final models for HMPV and RSV, respectively, were obtained by stepwise removing factors with $P > 0.1$. Testing for a possible covariance between variables such as prematurity and ≥ 1 chronic disease showed that both variables could be entered into the same models, but that it was not possible to include specific chronic diseases. The results from the final models were presented as odds ratios (OR) with 95% confidence intervals (CI) and *P*-values, and used for inference.

Missing data has been specified in Fig 1 and tables, and no imputation of missing data has been done. *P*-values < 0.05 (two-sided) were considered as statistically significant, and IBM SPSS Statistics 22 were used in all analyses.

Results

During nearly 9 years, we included 3,214 children out of 3,932 (81.7%) with acute RTI into our main study cohort (Fig 1). Among the 56.5% (1816/3214) of hospitalized children with LRTI, HMPV was detected in 9.4% (171/1816) and RSV in 47.3% (859/1816), while 0.6% (11/1816) had both HMPV and RSV (Fig 1). In total, 1,041 HMPV- and RSV-infected children were included in the present study (Fig 1). Their median age was 8.7 months (range 0.3–189.1 months) and the majority was younger than 5 years old (97.9%, 1019/1041). NPA were collected from 85.7% (892/1041) of these children within 24 hours after presentation, and within 48 hours in 96.0% (999/1041).

Children with LRTI and HMPV

Tables 1–3 and S1 Table summarize baseline characteristics and details of the hospital courses among HMPV-infected children (n = 171). The children had a median age of 17.2 months and the majority were boys (59%). Nearly every fourth child was born premature. Every third child had ≥ 1 chronic disease. Asthma was the most common chronic disease at 20%, while 13% had other chronic airway diseases. The children presented at the hospital at a median of 4.0 days after onset of the symptoms, which most often were a cough (91%) and fever (88%). Symptoms of breathing difficulties and clinical signs of respiratory difficulty, such as chest retractions and tachypnea, appeared in half or more. The median peak CRP level was slightly elevated at 35 mg/L, whereas one-third had otitis media. Sixty-nine children had bronchiolitis (40%), which was slightly more frequent than pneumonia, appearing in 61 (36%). Obstructive bronchitis, asthma exacerbation and unspecified LRTI were less common. The majority of the patients received several treatment modalities, in particular inhalations (91%) and oxygen (60%), while fewer received antibiotics (39%) and corticosteroids (33%). Twenty-three (13%) children received respiratory support, among whom the majority were admitted to the PICU. The median length of stay at the hospital was 4.0 days, and the median severity score was 1.0. HMPV was detected as a single virus in 106 out of 171 (62%) children, while 65 (38%) had HMPV with a co-detection of one or more viruses (rhinovirus (n = 27), enterovirus (n = 22), parainfluenza virus 1–4 (n = 9), human bocavirus (n = 8), adenovirus (n = 6), human parechovirus (n = 3) and influenza A and B viruses (n = 4)). All baseline characteristics, symptoms,

Table 1. Baseline Characteristics and Medical history in Hospitalized Children with Lower Respiratory Tract Infection, by Virus Type (HMPV vs RSV) and Infection Status (single virus infection vs co-detection).

	HMPV				RSV				HMPV vs RSV, *P*	
	Total (n = 171)	Single (n = 106)	Co-detection (n = 65)	*P*	Total (n = 859)	Single (n = 540)	Co-detection (n = 319)	*P*	Single (n = 646)	Co-detection (n = 384)
Age, months	17.2 (8–29)	14.7 (8–25)	18.5 (9–29)	0.253	7.3 (2–17)	5.4 (1–14)	12.5 (4–20)	<0.001	<0.001	<0.001
Age < 6 months	29 (17)	22 (21)	7 (11)	0.433[a]	382 (44)	283 (52)	99 (31)	<0.001[a]	<0.001[a]	0.001[a]
Age 6–11 months	34 (20)	20 (19)	14 (22)		153 (18)	96 (18)	57 (18)			
Age 12–23 months	54 (32)	31 (29)	23 (35)		215 (25)	101 (19)	114 (36)			
Age 24–59 months	43 (25)	25 (24)	18 (28)		98 (11)	52 (10)	46 (14)			
Age ≥ 60 months	11 (6)	8 (8)	3 (5)		11 (1)	8 (1)	3 (1)			
Gender, male	101 (59)	64 (60)	37 (57)	0.656	477 (56)	289 (54)	188 (59)	0.123	0.195	0.764
≥ 1 siblings	124/163	77/102	47/61	0.821	612/821	385/514	227/307	0.760	0.900	0.611
	(76)	(75)	(77)		(75)	(75)	(74)			
Day care	67/170	36/105	31	0.082	254/858	123/539	131	<0.001	0.013	0.324
	(39)	(34)	(48)		(30)	(23)	(41)			
Prematurity[b]	39 (23)	22 (21)	17 (26)	0.414	117 (14)	64 (12)	53 (17)	0.049	0.014	0.069
Asthma	35 (20)	20 (19)	15 (23)	0.508	97 (11)	46 (9)	51 (16)	<0.001	0.001	0.167
Other chronic airway disease	22 (13)	16 (15)	6 (9)	0.266	23 (3)	16 (3)	7 (2)	0.500	<0.001	0.012
Heart disease	12 (7)	9 (8)	3 (5)	0.539	28 (3)	21 (4)	7 (2)	0.177	0.072	0.383
Epilepsy	7 (4)	5 (5)	2 (3)	0.710	11 (1)	7 (1)	4 (1)	1.0	0.033	0.269
Cerebral palsy	14 (8)	11 (10)	3 (5)	0.182	13 (2)	9 (2)	4 (1)	0.777	<0.001	0.098
Other chronic disease[c]	15 (9)	9 (8)	6 (9)	0.868	38 (4)	19 (4)	19 (6)	0.093	0.033	0.404
≥ 1chronic disease	59 (35)	36 (34)	23 (35)	0.849	150 (17)	81 (15)	69 (22)	0.013	<0.001	0.018

Data presented as absolute numbers and percent in parenthesis, except from "Age, months", which is median and interquartile range (IQR) in parenthesis. Fractions are provided when sample size deviates from the given value.

[a]Comparing all age categories.

[b]Children born < 36 gestational weeks.

[c]Among single HMPV-infected as retinopathy (n = 1), endocrine disorder (n = 1), Down syndrome (n = 2), hereditary essential tremor (n = 1), congenital malformations (n = 2), muscular and neuromuscular disorder (n = 1+1). Among HMPV with co-detection as Down syndrome (n = 2), gastrointestinal disease (n = 2), endocrine disorder (n = 1) and neuromuscular disorder (n = 1). Among single RSV-infected as congenital malformations (n = 4), endocrine disorders (n = 2), Down syndrome and other syndromes (n = 4+6), Hemophilia A (n = 1) and gastrointestinal disease (n = 2). Among RSV with co-detection as unspecified psychomotor retardation (n = 3), endocrine disorders (n = 2), Down syndrome and other syndromes (n = 4+2), neuromuscular disorder (n = 1), long QT syndrome (n = 1), congenital malformations (n = 1), gastrointestinal and urinary tract disease (n = 4+1).

HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus.

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clinical findings, diagnoses and disease severity measures, such as a need for oxygen, length of hospital stay, a need for respiratory support and admission to the PICU, appeared at similar rates among both the children with a single virus infection and those with HMPV co-detection. Hence, the combined disease severity score was also equal.

Comparison of Children Infected with Different HMPV Genotypes

HMPV genotypes were available from 147 out of 171 (86%) children (S2 Table). Genotype B was most frequent, detected in 80 NPA (54%), of which 26 were B1 and 54 were B2. Genotype A was detected in 67 NPA (46%), of which 12 were A2a, 28 were A2b and 27 were A2 (unassigned). No samples were positive for A1. There were no differences between infections elicited by genotypes A and B in demographic characteristics, medical history and disease

Table 2. Clinical Details in Hospitalized Children with Lower Respiratory Tract Infection (LRTI), by Virus Type (HMPV vs RSV) and Infection Status (single virus infection vs co-detection).

	HMPV				RSV				HMPV vs RSV, *P*	
	Total (n = 171)	Single (n = 106)	Co-detection (n = 65)	*P*	Total (n = 859)	Single (n = 540)	Co-detection (n = 319)	*P*	Single (n = 646)	Co-detection (n = 384)
Tachypnea (admission)	83/165 (50)	52/102 (51)	31/63 (49)	0.825	470/809 (58)	281/504 (56)	189/305 (62)	0.083	0.377	0.060
Oxygen < 93% (admission)	43/164 (26)	26/101 (26)	17/63 (27)	0.860	162/842 (19)	95/530 (18)	67/312 (21)	0.207	0.067	0.339
Peak temp.[a], °C, median	38.6	38.6	38.6	0.824	38.2	38.1	38.5	<0.001	<0.001	0.288
(IQR)	(37.8–39.6)	(37.8–39.5)	(37.9–39.7)		(37.6–39.2)	(37.5–39.0)	(37.7–39.4)			
Peak CRP[b], median (IQR)	35 (10–83)	35 (10–88)	26 (10–75)	0.449	19 (6–55)	15 (5–45)	31 (8–70)	<0.001	<0.001	0.668
Peak WBC[c], mean (SD)	11.9 (4.7)	11.7 (4.7)	12.8 (5.4)	0.478	12.0 (5.1)	11.8 (4.9)	12.4 (5.4)	0.126	0.767	0.797
Chest X-ray, abnormal	120	74	46		445	263	182			
Perihilar infiltrates /hyperinflation/atelectasis	59 (49)	38 (51)	21 (46)	0.544[d]	259 (58)	164 (62)	95 (52)	0.033[d]	0.088[d]	0.428[d]
Localized/lobular infiltrates/ pleural effusion	61 (51)	36 (49)	25 (54)		186 (42)	99 (38)	87 (48)			
Ct < 28	92 (54)	56 (53)	36 (55)	0.743*	712/847 (84)	458/533 (86)	254/314 (81)	0.154*		
Ct 28–35	62 (36)	38 (36)	24 (37)		117/847 (14)	65/533 (12)	52/314 (17)			
Ct > 35	17 (10)	12 (11)	5 (8)		18/847 (2)	10/533 (2)	8/314 (3)			
Inhalations	155 (91)	94 (89)	61 (94)	0.260	824/858 (96)	521 (96)	303/318 (95)	0.385	0.001	0.543
Antibiotics	66 (39)	40 (38)	26 (40)	0.768	216/849 (25)	126/534 (24)	90/315 (29)	0.108	0.002	0.069
Intravenous fluid	44 (26)	32 (30)	12/64 (19)	0.099	179/848 (21)	109/533 (20)	70/315 (22)	0.541	0.027	0.539
Nasogastric feed tube	37 (22)	26/104 (25)	11/63 (17)	0.255	207/856 (24)	151/538 (28)	56/318 (18)	<0.001	0.522	0.977
Corticosteroids	57 (33)	37/104 (36)	20/64 (31)	0.565	219/850 (26)	115/536 (21)	104/314 (33)	<0.001	0.002	0.771
Otitis media	56 (33)	37 (35)	19 (29)	0.443	194 (23)	114 (21)	80 (25)	0.179	0.002	0.485
Pneumonia	61 (36)	36 (34)	25 (38)	0.619§	187 (22)	99 (18)	88 (28)	<0.001§	<0.001§	0.160§
Bronchiolitis	69 (40)	42 (40)	27 (42)		554 (64)	383 (71)	171 (54)			
Bronchitis/unspec. LRTI	17 (10)	13 (12)	4 (6)		28 (3)	18 (3)	10 (3)			
Asthma exacerbation	24 (14)	15 (14)	9 (14)	90 (10)	40 (7)	50 (16)	50 (16)			

Data presented as absolute numbers and percent in parenthesis, if otherwise not specified. Fractions are provided when sample size deviates from the given values.

[a]Temperature sampled from 168 HMPV (104 single and 64 co-detected) and from 794 RSV (499 single and 295 co-detected).

[b]CRP, C-reactive protein in mg/L, sampled from 170 HMPV (105 single and all 65 co-detected) and from 825 RSV (520 single and 305 co-detected).

[c]WBC, White blood cell count, sampled from 161 HMPV (102 single and 59 co-detected) and from 801 RSV (500 single and 301 co-detected).

[d]Comparing perihilar infiltrates/hyperinflation/atelectasis with localized/lobular infiltrates/pleural effusions.

*Comparing the three Ct, cycle threshold, categories.

§Comparing the four LRTI groups (pneumonia, bronchiolitis, obstructive bronchitis/unspecified LRTI and asthma exacerbation).

HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus; IQR, interquartile range.

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Table 3. Disease Severity measures in Hospitalized Children with Lower Respiratory Tract Infection, by Virus Type (HMPV vs RSV) and Infection Status (single virus infection vs co-detection).

	HMPV				RSV				HMPV vs RSV, *P*	
	Total (n = 171)	Single (n = 106)	Co-detection (n = 65)	*P*	Total (n = 859)	Single (n = 540)	Co-detection (n = 319)	*P*	Single (n = 646)	Co-detection (n = 384)
Oxygen treatment, any	102 (60)	64 (60)	38 (58)	0.804	542/857 (63)	351/539 (65)	191/318 (60)	0.138	0.351	0.810
Oxygen (days), median (IQR)[a]	4.0 (2.0–6.0)	3.0 (2.0–6.0)	4.0 (2.0–6.5)	0.571	3.0 (2.0–5.0)	3.0 (2.0–5.0)	3.0 (1.5–4.5)	0.572	0.073	0.022
Resp. support, any	23 (13)	15 (14)	8 (12)	0.732	108 (13)	77 (14)	31 (10)	0.056	0.977	0.529
Resp. support, non-invasive	21 (12)	14 (13)	7 (11)	0.637	105 (12)	75 (14)	30 (9)	0.053	0.852	0.734
Resp. support, invasive	8 (5)	5 (5)	3 (5)	1.0	12 (1)	11 (2)	1 (0)	0.038	0.160	0.016
PICU admission	20 (12)	14 (13)	6 (9)	0.432	90 (10)	65 (12)	25 (8)	0.052	0.737	0.707
Length of stay, median (IQR)	4.0 (2.0–6.0)	4.0 (2.8–6.0)	4.0 (2.0–6.0)	0.860	4.0 (2.0–6.0)	4.0 (2.0–6.0)	4.0 (2.0–6.0)	0.654	0.940	0.969
Length of stay $\geq$ 6 days	49 (29)	30 (28)	19 (29)	0.896	238 (28)	149 (28)	89 (28)	0.923	0.881	0.828
Severity score, median (IQR)	1.0 (0.0–2.0)	1.0 (0.0–2.0)	1.0 (0.0–2.0)	0.885	1.0 (0.0–2.0)	1.0 (0.0–2.0)	1.0 (0.0–2.0)	0.191	0.496	0.951
Severity score $\geq$ 2	48 (28)	30 (28)	18 (28)	0.931	242 (28)	158 (29)	84 (26)	0.357	0.843	0.821

Data presented as absolute numbers and percent in parenthesis, if otherwise not specified. Fractions are provided when sample size deviates from the given values.

[a]Data from 99 hMPV-infected

(61 single and 38 co-detected) and 525 RSV-infected (340 single and 185 co-detected).

HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus; IQR, interquartile range; PICU, pediatric intensive care unit.

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severity measures, as expressed by single variables and the combined disease severity score (S2 Table), though with one exception: Children infected with genotype B more often received respiratory support compared to children with genotype A (14/80, 18%, vs. 4/67, 6%, $P = 0.034$). However, when we only included single HMPV-infected in this analysis (n = 90), this difference disappeared (genotype B: 7/47, 15% vs. genotype A: 4/43, 9%, $P = 0.419$). Finally, when we compared subtypes B1 vs. B2, and A2a vs. A2b vs. A2 (unassigned), both as single virus infections and co-detections, no differences were found in disease severity score (data not shown).

Children with LRTI and RSV

Tables 1–3 and S1 Table summarize baseline characteristics and details of the hospital courses among RSV-infected children (n = 859). The children had a median age of 7.3 months, and the majority were boys (56%). Bronchiolitis was the most common diagnosis, appearing in nearly two-thirds, and approximately one-fifth had pneumonia. Single virus infection with RSV appeared in 540 out of 859 children (63%), while 319 out of 859 (37%) had RSV with the co-detection of other viruses (rhinovirus (n = 178), human parechovirus (n = 54), human bocavirus (n = 53), coronavirus (n = 46), adenovirus (n = 28), parainfluenza virus 1–4 (n = 12) and influenza A and B viruses (n = 11). Children with single virus infection were younger compared to those with RSV and co-detection (median 5.4 vs. 12.5 months, $P < 0.001$). Children with RSV infection and co-detection more often had fever before admission, whereas other symptoms appeared at similar rates in both RSV groups. Furthermore, more children with RSV and co-detection were born premature and had $\geq$ 1 chronic disease; they also

developed a higher peak temperature, higher peak CRP levels, more often had pneumonia and less often had bronchiolitis. There was a tendency that less children with RSV and co-detection needed respiratory support and admission to the PICU, whereas the need for oxygen, length of hospital stay and the combined disease severity score did not differ between the two RSV groups (Table 3).

Children Infected with Both HMPV and RSV

Eleven hospitalized children had both HMPV and RSV (Fig 1). They were diagnosed with pneumonia (n = 6), bronchiolitis (n = 2), asthma exacerbation (n = 2) and unspecified LRTI (n = 1), and their median age was 24.3 months. The median length of stay was five days (IQR 2.0–8.0) and the severity score median 1.0 (IQR 0.0–2.0). Seven out of 11 (64%) needed oxygen, but no received respiratory support. There were no differences in length of stay, need for oxygen and the combined disease severity score when comparing children with both HMPV and RSV (n = 11) with the entire groups of children with HMPV and RSV (n = 171 and n = 859, respectively).

Comparison of Children with LRTI Due to HMPV and RSV

Tables 1–4 show comparisons of baseline characteristics, medical details and details of the hospital courses of the children with HMPV and RSV. First, we compared single virus-infected children with HMPV and RSV (Table 1). The single HMPV-infected children were older (14.7 months vs. 5.4 months, $P < 0.001$), more often were born premature and more often had chronic diseases including asthma, other chronic airway diseases, epilepsy and cerebral paresis, compared to children with a single RSV infection (Table 1). Age-stratified analyses showed that these differences primarily appeared in children aged 12–23 months (prematurity) and 6–11 months (chronic diseases), respectively (Table 4). More children with a single HMPV infection had otitis media and pneumonia, and were treated with antibiotics and corticosteroids (Table 2). In contrast, more children with a single RSV infection had bronchiolitis and received inhalations (Table 2). However, when we controlled for age we found no significant differences in the distribution of diagnoses (Table 4). Interestingly, among children aged <6 months, nine out of 10 children with HMPV, as well as those with RSV, had bronchiolitis (Table 4). Children with a single HMPV infection had a higher peak temperature and higher peak CRP levels, but adjusted for age, these differences also disappeared (Tables 1 and 4). When we compared the entire single virus-infected groups with HMPV and RSV, there were no differences in disease severity, i.e. there were no differences in need of oxygen and respiratory support, PICU admission and length of hospital stay. The combined disease severity scores were also equal (Table 3). However, age was a strong factor for explaining the disease severity (Table 4 and Fig 2). The effect of age differed among HMPV and RSV infected children. In the youngest age group of <6 months old, the children with HMPV infection had a milder disease because they less frequently needed oxygen, and they had a shorter hospital stay and a lower severity score (Table 4 and Fig 2). On the other hand, the single HMPV-infected children in the age group of 12–23 months had a more severe disease than the children with RSV; they were treated more days with oxygen, more often received respiratory support, more often were admitted to the PICU, had a longer hospital stay and more often had a severity score ≥2 (Table 4 and Fig 2).

We also compared the children with a co-detection of other viruses, in addition to HMPV and RSV (HMPV: n = 65 and RSV: n = 319) (Tables 1–3 and S1 Table). We retrieved the age difference seen in single virus-infected children, although it was less pronounced (HMPV: 18.5 months vs. RSV: 12.5 months, $P < 0.001$) (Table 1). More children with HMPV and co-

Table 4. Clinical Characteristics in Hospitalized children with Lower Respiratory Tract Infection (LRTI), by Age and Single Virus Type (HMPV: n = 106 and RSV: n = 540).

	< 6 months			6–11 months			12–23 months			≥ 24 months		
	HMPV (n = 22)	RSV (n = 283)	*P*	HMPV (n = 20)	RSV (n = 96)	*P*	HMPV (n = 31)	RSV (n = 101)	*P*	HMPV (n = 33)	RSV (n = 60)	*P*
Premature born[a]	2 (9)	24 (8)	1.0	3 (15)	12 (13)	0.721	11 (35)	13 (13)	0.004	6 (18)	15 (25)	0.452
≥ 1 chronic disease	0 (0)	12 (4)	1.0	7 (35)	7 (7)	0.003	12 (39)	29 (29)	0.293	17 (52)	33 (55)	0.747
Peak temp.[b]**, median**	37.9	37.8	0.431	38.4	38.4	0.692	39.3	39.0	0.113	38.8	39.0	0.567
(IQR)	(37.4–38.6)	(37.4–38.2)		(37.6–39.9)	(37.9–39.2)		(38.3–39.8)	(38.1–39.6)		(38.0–39.5)	(38.0–39.7)	
Peak CRP[c]**, median**	18	9	0.219	27	15	0.270	31	32	0.987	76	42	0.094
(IQR)	(4–45)	(0–27)		(8–95)	(6–55)		(8–82)	(10–75)		(28–118)	(14–96)	
Otitis media	2 (9)	28 (10)	1.0	7 (35)	33 (34)	0.957	13 (42)	38 (38)	0.666	15 (45)	15 (25)	0.043
Pneumonia	1 (5)	25 (9)	1.0*	7 (35)	15 (16)	NA§	13 (42)	29 (29)	0.373#	15 (45)	30 (50)	0.675†
Bronchiolitis	20 (91)	256 (90)		8 (40)	69 (72)		14 (45)	58 (57)		0 (0)	0 (0)	
Other diagnoses[d]	1 (5)	2 (1)		5 (25)	12 (13)		4 (13)	14 (14)		18 (55)	30 (50)	
Oxygen treatment	4 (18)	177 (63)	<0.001	12 (60)	55/95 (58)	0.862	25 (81)	71 (70)	0.258	23 (70)	48 (80)	0.263
Oxygen days‡, median	3.0	3.0	0.888	2.0	2.5	0.670	4.0	2.0	0.010	4.5	4.0	0.822
(IQR)	(1.5–4.5)	(2.0–5.0)		(1.0–4.3)	(1.0–4.0)		(3.0–7.0)	(2.0–4.0)		(2.0–6.8)	(2.0–6.3)	
Resp. support, any	2 (9)	59 (21)	0.269	2 (10)	7 (7)	0.652	6 (19)	6 (6)	0.034	5 (15)	5 (8)	0.318
Resp. support, non-invasive	2 (9)	59 (21)	0.269	2 (10)	6 (6)	0.625	6 (19)	6 (6)	0.034	4 (12)	4 (7)	0.448
Resp. support, invasive	0 (0)	5 (2)	1.0	1 (5)	2 (2)	0.436	3 (10)	3 (3)	0.141	1 (3)	1 (2)	1.0
PICU admission	2 (9)	51 (18)	0.390	2 (10)	6 (6)	0.625	6 (19)	4 (4)	0.011	4 (12)	4 (7)	0.448
Length of stay: median	3.0	4.0	0.018	4.0	3.0	0.240	4.0	3.0	0.027	3.0	4.0	0.469
(IQR)	(1.8–4.0)	(3.0–6.0)		(3.0–5.0)	(2.0–5.0)		(3.0–8.0)	(2.0–5.0)		(2.0–7.5)	(3.0–7.0)	
Length of stay ≥ 6 days	3 (14)	81 (29)	0.130	2 (10)	21 (22)	0.356	13 (42)	23 (23)	0.036	12 (36)	24 (40)	0.730
Severity score: median	0.0	1.0	0.001	1.0	1.0	0.869	1.0	1.0	0.062	1.0	1.0	0.652
(IQR)	(0.0–0.3)	(0.0–2.0)		(0.0–1.0)	(0.0–1.0)		(1.0–2.0)	(1.0–1.0)		(0.0–2.0)	(1.0–2.0)	
Severity score ≥ 2	2 (9)	96 (34)	0.016	3 (15)	19 (20)	0.761	13 (42)	19 (19)	0.009	12 (36)	24 (40)	0.730

Data presented as absolute numbers and percent in parenthesis, if otherwise not specified. Fractions are provided when the sample size deviates from the given values.

[a]Born < 36 gestational weeks.

[b]Peak temperature in ˚C sampled from 104 HMPV-infected and 499 RSV-infected.

[c]CRP, C-reactive protein, sampled from 105 HMPV-infected and 520 RSV-infected.

[d]The sum of children with obstructive bronchitis/unspecified LRTI and asthma exacerbation.

*Comparing pneumonia and bronchiolitis.

§NA, not applicable, when comparing pneumonia, bronchiolitis and other diagnoses.

#Comparing pneumonia, bronchiolitis and other diagnoses.

†Comparing pneumonia and other diagnoses.

‡Days with oxygen sampled from 61 out of the 64 HMPV-infected, and from 340 out of the 351 RSV-infected, whom needed oxygen.

HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus; IQR, interquartile range; PICU, pediatric intensive care unit.

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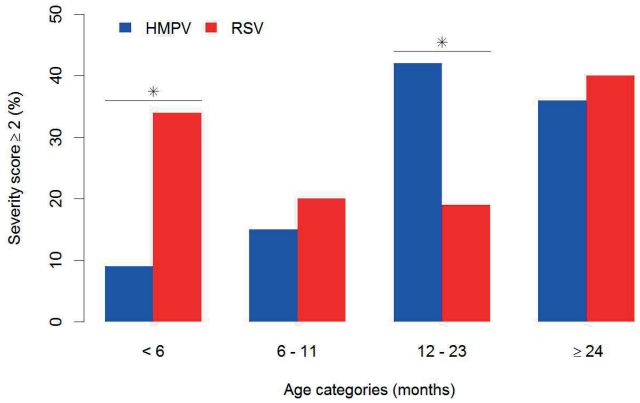

Fig 2. Proportions of children (%) with severe lower respiratory tract infection, severity score ≥ 2, among single virus-infected children with HMPV (blue) (n = 106) and RSV (red) (n = 540), according to age categories. Asterisk indicates significant differences ($P < 0.05$).

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detection had one or more chronic diseases, including chronic airway diseases other than asthma. There were no differences in peak temperature, peak CRP levels and LRTI diagnoses. The clinical courses also differed to some extent (Table 3). Although the same rate of children with HMPV and RSV co-detections needed oxygen, HMPV-positive children needed oxygen longer ($P = 0.022$). Invasive respiratory support was also given more often to HMPV-infected children ($P = 0.016$), whereas non-invasive respiratory support was given at similar rates (Table 3).

Two children died because of RTI during the entire nine-year-long study period. One was only 2.5 months old and had a single RSV infection. The other child was two years old and had a single HMPV infection. Both had severe comorbidities.

Risk Factors for Severe Disease Due to HMPV and RSV

In the logistic regression analyses we found that age was strongly associated with disease severity (Table 5). In HMPV-infected children, age groups 12–23 months (OR = 3.01, $P = 0.067$) and ≥24 months (OR = 3.97, $P = 0.021$) were associated with the highest risk for severe disease, while in the youngest age group (<6 months) RSV-infected children had the highest risk for severe disease (OR = 2.11, $P = 0.002$). Prematurity was associated with higher risk in both HMPV- (OR = 3.36, $P = 0.005$) and RSV-infected children (OR = 1.58, $P = 0.035$). Chronic disease was also an important factor, being significantly associated with higher risk in RSV-infected children (OR = 2.26, $P < 0.001$) and close to significant in HMPV-infected children (OR = 2.22, $P = 0.059$). High viral load was associated with higher risk for severe disease in RSV-infected children only (OR = 7.91, $P = 0.047$) and not in those with HMPV. No significant interactions were present among variables included in the two final models, respectively. Finally, viral co-detection was not associated with increased risk for severe disease, and this factor was not included in the final models for HMPV and RSV. Other factors, not included in the predefined models, such as genotype (HMPV), gender, siblings and day care attendance, were also analyzed with logistic regression, but none of these factors yielded any significant contributions (data not shown).

Table 5. Results from Final Models[a], presenting Odds Ratios (OR) with 95% Confidence Intervals (95% CI) and *P*-values, for the Associations between Risk Factors and Severe Lower Respiratory Tract Infection, by Virus Type (HMPV or RSV).

Risk factor	HMPV (n = 171)		RSV (n = 847)	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age < 6 months	1.16 (0.23–5.87)	0.853	2.11 (1.33–3.35)	0.002
Age 6–11 months	1.0		1.0	
Age 12–23 months	3.01 (0.93–9.75)	0.067	1.19 (0.70–2.00)	0.525
Age ≥ 24 months	3.97 (1.24–12.73)	0.021	1.34 (0.72–2.52)	0.360
Premature[b] born	3.36 (1.44–7.83)	0.005	1.58 (1.03–2.42)	0.035
Not premature born	1.0		1.0	
≥ 1 chronic disease	2.22 (0.97–5.09)	0.059	2.26 (1.44–3.54)	< 0.001
No chronic disease	1.0		1.0	
Ct[c] < 28	. . .		7.91 (1.03–61.05)	0.047
Ct 28–35	. . .		5.33 (0.67–42.59)	0.115
Ct > 35	. . .		1.0	

[a]Final binary logistic regression models, in which HMPV and RSV are analyzed separately. Full models included all factors as age, prematurity, chronic disease, Ct-values and viral co-detection. Factors were stepwise removed when $P > 0.1$.

[b]Born < 36 gestational weeks.

[c]Cycle threshold values, a measure of viral load. This factor was not included in the final model for HMPV.

HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus.

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Discussion

The present data from our large population-based study collected during a nearly 9-year-long period show that LRTI with HMPV clinically manifests itself independently of the co-detection of other viruses, and does not differ in relation to HMPV genotypes. Furthermore, clinical manifestations and final diagnoses in children with HMPV and RSV LRTI are quite similar. However, the clinical course varies in relation to age, and the age effect differed among single virus HMPV- and RSV-infected children. Lastly, our data confirm that hospitalized children born preterm and children with chronic diseases have an increased risk of developing severe LRTI among HMPV- and RSV-infected.

Using a broad panel of sensitive nucleic-acid based viral tests, we detected more than one virus in 38% of the children with LRTI and HMPV. All patient characteristics, including rates of prematurity and chronic diseases, clinical manifestations and clinical courses were surprisingly equal in those with single HMPV infection and HMPV with viral co-detection. On this basis, and on observations done by others [14,17,23], it seems evident to conclude that viral co-detections in HMPV-infected children usually have no cumulative clinical effects to that of HMPV alone. Our population-based data also clearly shows that HMPV/RSV-coinfection is a rare event, and is usually not associated with increased severity, as has been suggested from smaller studies based on selected groups of children [21,22].

We detected a broad spectrum of HMPV genotypes (A2, A2a, A2b, B1 and B2), but no samples were positive for genotype A1. In other studies, genotype A1 has also been the most seldom genotype detected [13–15,17,36]. Our data clearly show that the present HMPV genotype variations do not relate to any particular premorbid condition, and causes infection with quiet similar clinical manifestations and outcomes in children. Consequently, our findings confirm results from previous studies with smaller sample sizes [13–15,36,37]. However, Schuster et al. [17] genotyped 192 HMPV-positive in children <2 years old in Jordan enrolled during three years, and found that HMPV genotype A infection was associated

with an increased need for oxygen compared to genotype B infection. In another study, including 68 HMPV-positive hospitalized children <3 years of age enrolled during four winter seasons in Canada by Papenburg et al. [18], infection with HMPV genotype B was associated with either an increased oxygen need, PICU attendance or a hospitalization >5 days. The diverging findings in these two studies compared to our study may be explained by different age of the included children, and because the outcome "severe disease" in the two other studies probably included less ill children than in our study. Furthermore, naturally occurring genotype variations over shorter time intervals may also increase the risk of random findings.

The most prominent factor differing between HMPV and RSV in the present study was the difference in age distributions, which has been observed before [18,23,29,38,39]. Two-thirds with HMPV infection were 1 to 5 years old and less than one-fifth was <6 months old, and nearly half of RSV-infected children were <6 months old. In addition, we confirmed findings from previous studies that more HMPV-infected children were preterm born [18,29] and had a chronic disease [2,32]. On the other hand, HMPV and RSV in many ways caused a quite similar spectrum of LRTI types. Looking at the entire groups of children with HMPV and RSV infections, bronchiolitis was the most common diagnosis in both viruses, although a 50% higher rate was observed in children with RSV. By contrast, HMPV-infected children apparently had pneumonia and otitis media more often. However, these findings were confounded by age. In children <6 months old with a single virus infection, 90% of both viral infections were classified as bronchiolitis, and in children older than 6 months, no significant differences in bronchiolitis and pneumonia rates were found. In previous studies, it has been shown that temperature and CRP may increase to higher levels in children with HMPV infection than in those with RSV [3,38], but we could not confirm this after adjusting for age.

We found that disease severity was very similar when we compared the entire groups of children with HMPV and RSV, and others have also previously reported this [23,25,26]. However, age was strongly related to disease severity, and the age effect differed among single virus HMPV- and RSV-infected children. First of all, only one-fifth of the hospitalized children with single HMPV infection were younger than 6 months, compared to half of those with RSV. Secondly, HMPV infection was associated with a milder disease than RSV infection among children aged <6 months, as indicated by a less frequent need for oxygen, a shorter hospital stay and a lower severity score. Furthermore, the data provided evidence that in children aged 12–23 months old, HMPV infection may be more severe than RSV infection, with a longer need for oxygen treatment, more children in need of respiratory support, more children admitted to PICU, a longer hospital stay and a higher severity score. A possible explanation for these observations might have been that neonates attain higher concentrations of maternally derived protective antibodies against HMPV, as compared to RSV, during pregnancy and the first six months of life. However, data from a recent clinical study measuring HMPV and RSV antibody concentrations did not confirm this hypothesis [40]. Another explanation might have been that HMPV-infected children more often than children with RSV had a primary and potentially more severe infection among those children aged 12–23 months, but it was not possible for us to assess whether children had a primary or secondary infection. In general, clinical manifestations in children with airway infections are related to the net effect of physical and genetic factors, as well as viral- and immune-mediated reactions in the maturing child, which are strongly correlated to the child's age [41,42]. We found that high RSV viral loads, but not high HMPV viral loads, were associated with severe disease. Thus, based on these clinical observations among our population of hospitalized children, it may be tempting to claim that RSV is a more potent virus than HMPV

among infants, and that RSV infection more than HMPV is a virally driven disease. Recently, other researchers have published data supporting a similar assumption [43]. In accordance with our findings, Roussy et al. [37] found that HMPV viral loads were not associated with increased disease severity among hospitalized children (inpatients), but hospitalized patients had higher HMPV viral loads than outpatients [37]. It has also been shown by others that LRTI may be associated with higher HMPV viral loads than URTI [44]. For this reason, it seems that HMPV viral loads may relate to disease severity to a certain extent, but not among those with the most severe disease.

Most previous studies on risk factors for severe HMPV infections in children focused on age groups younger than 2–3 years old [17,18], high-risk patients [29] or for children admitted to PICU [33], and disease severity has been defined by the use of various outcome variables [17,18,29,33]. We included a population-based sample consisting of all children aged <16 years who were admitted with acute RTI, although the vast majority were aged <5 years. We used a compound severity score combining several outcome measures. Although this score has not been validated, it fit the routines at our department and rather rigorously defined severe disease, and provided reliable risk factor estimates. We confirmed that independent risk factors for both severe HMPV and RSV infections were the presence of chronic diseases and a history of prematurity. Children aged 12 to 23 months had a three-fold increased risk of developing severe HMPV infection, and those aged ≥24 months had a nearly four-fold increased risk. Among RSV-infected children, infants less than six months had a nearly double risk compared to older children. Having one or more chronic diseases doubled the risk in both virus types, but due to a significant co-variation, our data set could not be used to identify which chronic diseases more precisely increased the risk. Prematurity with a gestational age less than 36 weeks increased the risk of severe HMPV infection three-fold, as shown by others [31], and severe RSV infection for approximately 50%. However, prophylactic use of palivizumab in high-risk children may have confounded this risk estimation in relation to RSV. Hence, in hospitalized children, our data confirm the findings from other studies that particular age groups, prematurity and the presence of chronic diseases independently increase the risk of developing severe LRTI among children with HMPV infection [2,17,18,29,31–33] and RSV infection [18,24,27–30].

It is a strength of the present study that we prospectively enrolled children of all ages from the same county in Mid-Norway, and to the only existing hospital in this region during a nearly 9-year long period. NPA were taken from the majority of the admitted children, and 81.7% were included in the main study cohort. Moreover, we analyzed all NPA using a broad panel of sensitive virus tests during the entire period, which allowed us to examine viral co-detections thoroughly. Nonetheless, it may be a limitation that bacterial co-detections were not considered, but most children had low or moderately increased CRP values. Furthermore, during the entire study period almost all Norwegian children received conjugated pneumococcal vaccines, which has reduced the incidence of pneumococcal infections [45]. Although this does not completely exclude pneumococcal coinfection, at least HMPV- and RSV-infected children may have been similarly influenced. Diagnostic and work-up biases could have affected our results negatively, since the clinicians were not blinded for the NPA results, and because patients were not treated after a study protocol.

In conclusion, HMPV infections among hospitalized children with LRTI were manifested independently of viral co-detection and HMPV genotypes. HMPV and RSV infections differed clinically to a certain extent, and these differences were mostly related to age. Among single virus-infected children, HMPV-infected aged <6 months had a milder disease and those aged 12–23 months had more severe disease, than children with RSV. A history of prematurity and chronic disease increased the risk of severe LRTI among HMPV- and RSV-infected children.

Supporting Information

S1 Table. Symptoms, Clinical findings at Admission and Upper Respiratory Tract Infection Diagnoses in Hospitalized Children with Lower Respiratory Tract Infection, by Virus Type (HMPV vs RSV) and Infection Status (single virus infection vs co-detection). Data presented as absolute numbers and percent in parenthesis, except from symptom-days as median with interquartile range, IQR, in parenthesis. Fractions are provided when samples size deviates from the given value. *Data from 163 HMPV-infected (102 single and 61 co-detected) and 831 RSV-infected (519 single and 312 co-detected). HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus.
(DOCX)

S2 Table. Medical history, Clinical Details and Disease Severity measures in 147 children with Lower Respiratory Tract Infection (LRTI), by HMPV genotype A vs B. Data presented as absolute numbers and percent in parenthesis, if otherwise not specified. *CRP, C-reactive protein, sampled from all HMPV A and 79 HMPV B. †WBC, White blood cell count, sampled from 60 HMPV A and 78 HMPV B. ‡NA, not applicable, when comparing the three Ct categories. §Comparing the four LRTI groups (pneumonia, bronchiolitis, obstructive bronchitis/unspecified LRTI and asthma exacerbation). HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus; IQR, interquartile range, GA, gestational age; Ct-values, cycle threshold values.
(DOCX)

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S1 Table. Symptoms, Clinical findings at Admission and Upper Respiratory Tract Infection Diagnoses in Hospitalized Children with Lower Respiratory Tract Infection, by Virus Type (HMPV vs RSV) and Infection Status (single virus infection vs co-detection).

	HMPV				RSV				HMPV vs RSV, *P*	
	Total (n = 171)	**Single (n = 106)**	**Co-detection (n = 65)**	***P***	**Total (n = 859)**	**Single (n = 540)**	**Co-detection (n = 319)**	***P***	**Single (n = 646)**	**Co-detection (n = 384)**
Cough	155 (91)	93 (88)	62 (95)	0.095	762 (89)	475 (88)	287 (90)	0.370	0.948	0.167
Fever	150 (88)	93 (88)	57 (88)	0.993	591 (69)	356 (66)	235 (74)	0.018	<0.001	0.016
Heavy breathing	114 (67)	70 (66)	44 (68)	0.824	651 (76)	406 (75)	245 (77)	0.679	0.041	0.121
Stuffed nose	94 (55)	57 (54)	37 (57)	0.688	564 (66)	362 (67)	202 (63)	0.268	0.009	0.332
Reduced appetite	88 (51)	61 (58)	27 (42)	0.042	519 (60)	326 (60)	193 (61)	0.970	0.588	0.005
Wheezing	62 (36)	37 (35)	25 (38)	0.639	396 (46)	247 (46)	149 (47)	0.783	0.039	0.223
Trout pain	34 (20)	21 (20)	13 (20)	0.976	164 (19)	102 (19)	62 (19)	0.844	0.825	0.917
Ear pain	21 (12)	13 (12)	8 (12)	0.993	70 (8)	42 (8)	28 (9)	0.605	0.130	0.373
Apnea	1 (1)	0 (0)	1 (2)	0.380	36 (4)	25 (5)	11 (3)	0.404	0.023	0.699
Symptom-days* before admission	4.0 (3.0-5.0)	4.0 (3.0-5.0)	4.0 (2.0-5.0)	0.252	4.0 (3.0-5.0)	4.0 (3.0-5.0)	4.0 (2.0-5.75)	0.700	0.059	0.798
Abnormal tympanic membrane	55/169 (33)	37/104 (36)	18 (28)	0.287	190 (22)	111 (21)	79 (25)	0.151	<0.001	0.621
Pharyngitis	57/169 (34)	36/104 (35)	21 (32)	0.758	299 (35)	178 (33)	121 (38)	0.140	0.743	0.392
Rhinitis	45/169 (27)	28/104 (27)	17 (26)	0.912	280 (33)	174 (32)	106 (33)	0.761	0.286	0.265
Tonsillitis	21/169 (12)	14/104 (13)	7 (11)	0.606	70 (8)	35 (6)	35 (11)	0.020	0.014	0.962
Retractions at inspection	115/170 (68)	74/105 (70)	41 (63)	0.316	635 (74)	404 (75)	231 (72)	0.439	0.353	0.131
Expiratory wheezing (auscultation)	69/170 (41)	36/105 (34)	33 (51)	0.033	384 (45)	243 (45)	141 (44)	0.820	0.043	0.332
Rales (auscultation)	70/170 (41)	44/105 (42)	26 (40)	0.806	387 (45)	256 (47)	131 (41)	0.071	0.301	0.873
Crepitation (auscultation)	59/170 (35)	37/105 (35)	22 (34)	0.853	280 (33)	169 (31)	111 (35)	0.290	0.428	0.883
Otitis media	56 (33)	37 (35)	19 (29)	0.443	194 (23)	114 (21)	80 (25)	0.179	0.002	0.485
Rhino-pharyngitis	97 (57)	62 (58)	35 (54)	0.552	502 (58)	307 (57)	195 (61)	0.219	0.755	0.272
Tonsillitis	21 (12)	14 (13)	7 (11)	0.637	71 (8)	35 (6)	36 (11)	0.013	0.017	0.904
Acute laryngitis	1 (1)	1 (1)	0 (0)	1.0	5 (1)	3 (1)	2 (1)	1.0	0.513	1.0

Data presented as absolute numbers and percent in parenthesis, except from symptom-days as median with interquartile range, IQR, in parenthesis. Fractions are provided when samples size deviates from the given value. *Data from 163 HMPV-infected (102 single and 61 co-detected) and 831 RSV-infected (519 single and 312 co-detected).

HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus.

S2 Table. Medical history, Clinical Details and Disease Severity measures in 147 children with Lower Respiratory Tract Infection (LRTI), by HMPV genotype A vs B.

	HMPV A (n = 67)	HMPV B (n = 80)	*P*
Age, months, median (IQR)	14.7 (9-24)	18.5 (7-33)	0.295
Gender, male	40 (60)	49 (61)	0.848
Prematurity (GA < 36 weeks)	14 (21)	17 (21)	0.958
≥ 1 chronic disease	23 (34)	24 (30)	0.575
Peak CRP*, median (IQR)	29 (9-61)	35 (10-79)	0.511
Peak WBC†, mean (SD)	10.9 (4.3)	12.5 (4.6)	0.042
Ct < 28	42 (63)	43 (54)	NA‡
Ct 28-35	21 (31)	36 (45)	
Ct > 35	4 (6)	1 (1)	
Viral co-detection	24 (36)	33 (41)	0.501
Pneumonia	20 (30)	28 (35)	0.336§
Bronchiolitis	34 (51)	29 (36)	
Obstructive bronchitis and unspecific LRTI	5 (7)	9 (11)	
Asthma exacerbation	8 (12)	14 (18)	
Oxygen treatment, any	45 (67)	45 (56)	0.176
Respiratory support	4 (6)	14 (18)	0.034
PICU admission	4 (6)	11 (14)	0.121
Length of stay, median (IQR)	4.0 (3.0-6.0)	4.0 (2.0-6.0)	0.915
Severity score, median (IQR)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.788
Severity score ≥ 2	18 (27)	22 (28)	0.931

Data presented as absolute numbers and percent in parenthesis, if otherwise not specified. *CRP, C-reactive protein, sampled from all HMPV A and 79 HMPV B. †WBC, White blood cell count, sampled from 60 HMPV A and 78 HMPV B. ‡NA, not applicable, when comparing the three Ct categories. §Comparing the four LRTI groups (pneumonia, bronchiolitis, obstructive bronchitis/unspecific LRTI and asthma exacerbation).
HMPV indicates human metapneumovirus; RSV, respiratory syncytial virus; IQR, interquartile range, GA, gestational age; Ct-values, cycle threshold values.

Paper III

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RESEARCH ARTICLE

Respiratory Virus Detection and Clinical Diagnosis in Children Attending Day Care

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Data Availability Statement: The data will not be uploaded in the manuscript, supplemental files or in a public repository because of ethical and legal restrictions. The study was approved by the Regional Committee for Medical and Health Research Ethics (REK), Mid-Norway, Norway (no.2011/2246). Due to legal restrictions from REK we cannot make the data publicly available. Nina Moe, nina.moe@ntnu.no, might be contacted and will distribute anonymous data upon request to interested researchers.

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Abstract

Background

Respiratory viruses often have been studied in children with respiratory tract infection (RTI), but less knowledge exists about viruses in asymptomatic children. We have studied the occurrence of a broad panel of respiratory viruses in apparently healthy children attending day care, taking into account the influence of possible confounding factors, such as age, clinical signs of respiratory tract infection (RTI), location (day-care section) and season.

Methods

We have studied 161 children in two day-care centers, each with separate sections for younger and older children, during four autumn and winter visits over a two-year period. A total of 355 clinical examinations were performed, and 343 nasopharyngeal samples (NPS) were analyzed by semi-quantitative, real-time, polymerase chain reaction (PCR) tests for 19 respiratory pathogens.

Result

Forty-three percent of all NPS were PCR-positive for $\geq$ 1 of 13 virus species, with high species variation during visits. Rhinovirus 26% (88/343 NPS), enterovirus 12% (40/343) and parechovirus 9% (30/343) were detected in every visit, and the rates varied in relation to age, day-care section and season. Ten other viruses were detected in $\leq$ 3% of the NPS. Generally, viruses occurred together in the NPS. In 24% (79/331) of the clinical examinations with available NPS, the children had clear signs of RTI, while in 41% (135/331) they had mild signs, and in 35% (117/331) the children had no signs of RTI. Moreover, viruses were found in 70% (55/79) of children with clear signs of RTI, in 41% (55/135) with mild signs and in 30% (35/117) without any signs of RTI ($p < 0.001$).

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Conclusions

Positive PCR tests for respiratory viruses, particularly picornaviruses, were frequently detected in apparently healthy children attending day care. Virus detection rates were related to age, presence of clinical signs of RTI, location in day care and season.

Introduction

The use of sensitive molecular tests such as polymerase chain reaction (PCR) has shown that several respiratory viruses frequently been detected in children who need hospitalization for respiratory tract infection (RTI) [1,2]. It has also been documented that children hospitalized with RTI often have multiple viruses, and that asymptomatic hospital controls may frequently be positive for respiratory viruses [3–6]. Outside the hospital setting, evidence exists for the presence of both well-known and recently detected viruses, such as respiratory syncytial virus (RSV), human rhinovirus (HRV), human metapneumovirus (hMPV) and human bocavirus (HBoV), in children with RTI [7,8]. However, it is more surprising that even asymptomatic children outside hospitals may harbor viruses in their airways, as has been recently shown [9–11]. We aim to study this phenomenon further, and describe the occurrence of a broad panel of respiratory pathogens in healthy children. Since nearly all Norwegian children attend day care on a daily basis, we have studied a group of apparently healthy children attending day care, taking into account the influence of possible confounding factors such as age, clinical signs of RTI, location in day care and season.

Materials and Methods

Study Population

The study was performed during four visits between March 2012 and February 2014 in two day-care centers in the city of Trondheim, Norway, with 95% of all toddlers and preschool children in Trondheim attended day care during the study period (Statistics Norway 2014). Norwegian children start school at the age of six, and the children included were between the ages of 1–6.3 years. The number of children in the two day-care centers varied from 110 to 132 at each visit. The children were organized into five or six sections (the number differed during the two years), with 6–12 of the youngest children per section, aged 1–3.8 years, and four sections for the oldest with 16–18 children per section, aged 2.8–6.3 years. In total, 161 children participated in the study one or more times (median two times, range one to four), which resulted in 368 out of 484 possible (76.0%) inclusions (Fig 1). The majority of included children was both sampled by a nasopharyngeal sample (NPS) and underwent clinical examination, although some resisted the collection of NPS or clinical examination after inclusion. With the exception of one, all children usually stayed 41 hours per week in the day-care center. The inclusion criterion was informed written consent from parents or guardians on behalf of the children for each study visit. Each child could be included only once during each study visit. The exclusion criterion was previous nasal bleeding. At each inclusion, the parents answered a form of baseline demographics, household characteristics and medical history. One of four pediatricians conducted a standardized clinical examination of each child during daytime in the day-care area. The pediatricians classified the children into three groups based on clinical findings: 1. No RTI with normal findings, 2. Mild RTI with discrete signs of rhinitis, pharyngitis, simplex media otitis or secretory media otitis, and 3. Clear RTI with significant signs of rhino-pharyngitis,

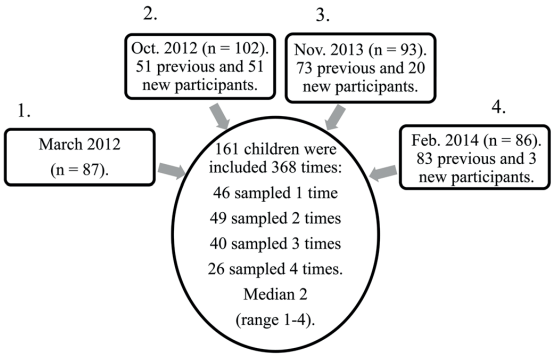

Fig 1. Design of the study. Number of included children during each of four study visits and the number of children being sampled one, two, three or four times.

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tonsillitis, purulent media otitis or auscultatory findings from the lower airways. The study was approved by the Regional Committee for Medical and Health Research Ethics, Mid-Norway, Norway (no. 2011/2246).

Sampling and Microbiologic Analyses

Nasopharyngeal samples were obtained by flocked swabs (Copan Italia SpA®) and placed immediately into a 3 ml transport medium (UTM-RT, Copan Italia SpA®). Samples were analyzed at the Department of Medical Microbiology, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway. In total, 361 NPS were collected, though some samples of poor quality (n = 18) were excluded. The NPS were analyzed with semi-quantitative, real-time PCR for 19 respiratory pathogens including human adenovirus (HAdV), HBoV, human coronavirus (HCoV) OC43, 229E, NL63, human enterovirus (HEV), human parechovirus (HPeV), hMPV, influenza A virus, influenza B virus, parainfluenza virus (PIV) types 1–4, RSV, HRV, *Bordetella pertussis*, *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. The PCRs were in-house, real-time assays with TaqMan probes [12]. The amount of virus in each sample was recorded semi-quantitatively, and based on the cycle threshold value (Ct-value). Ct-values above 40 were regarded as virus-negative.

Statistical Analyses

The χ^2 or a Fisher's Exact Test were used to compare differences in proportions, and continuous but not normally distributed data were analyzed by use of a Kruskal-Wallis test. The Monte Carlo simulation test described by Hope was used to test whether respiratory pathogens occurred independently of each other among children, using the algorithm by Patefield [13,14]. The test compared the observed distribution of the number of pathogens in a nasopharyngeal sample, with a distribution based on the assumption that pathogens occurred independently of each other and conditional on their observed frequencies. The test was based on 2,000 simulations of the null hypothesis. Following the rejection of the null hypothesis (see Results), the same approach was subsequently used to test whether the distribution of pathogens among day-care sections and sampling times could account for the general tendency of respiratory pathogens to occur together in NPS. In addition, in the latter test, the null distribution was conditional on the distribution of pathogens among day-care sections and sampling times. Hope's test was further used to test in pairs whether the three most common pathogens, HEV, HPeV

and HRV, occurred independently of each other. The sequential Bonferroni method was also used to control the familywise Type I error rate in these three tests [15]. The occurrence in NPS of the same three respiratory pathogens was analyzed in an explorative manner using generalized linear mixed-effect models with logit link functions [16]. Day-care sections and sampling times (seasons) were included in the logistic models as random explanatory variables, while the children's age in months and the occurrence of other viruses (coded as a binary variable) were included as fixed variables. The "top-down" approach recommended by Diggle et al. was followed, in which the random part of models was first determined based on the "beyond optimal model", before obtaining the minimal adequate model by selecting among the candidate's fixed parts [17,18]. Model selection was based on the Akaike information criterion (AIC) [19]. The same approach was followed in order to study whether clinical findings were related to the occurrence of HRV, which was the virus most frequently found in the NPS. Day-care sections and sampling times (seasons) were again included as random explanatory variables, whereas the occurrence of HRV and children's age were included as fixed variables. The response variable was the occurrence of clear findings of RTI coded as a binary variable, with mild and no RTI findings as the reference category. Moreover, statistically significant values were defined as $p<0.05$ (two-sided), and IBM SPSS Statistics 22 and R version 3.2.2 were used in the statistical analyses [20]. The R-package lme4 was used in the GLMM-modelling [21].

Results

Viral Findings

NPS were collected in 343 out of the 368 inclusions (93.2%). Overall, 149 (43%) of the samples were PCR-positive for virus, varying from 34% (25/74) to 56% (55/99) at each study visit (Table A in S1 File). There was a large variation in pathogen detections during the four visits (Fig 2), and only HEV, HPeV, and HRV were detected at all visits. HRV was the most frequent,

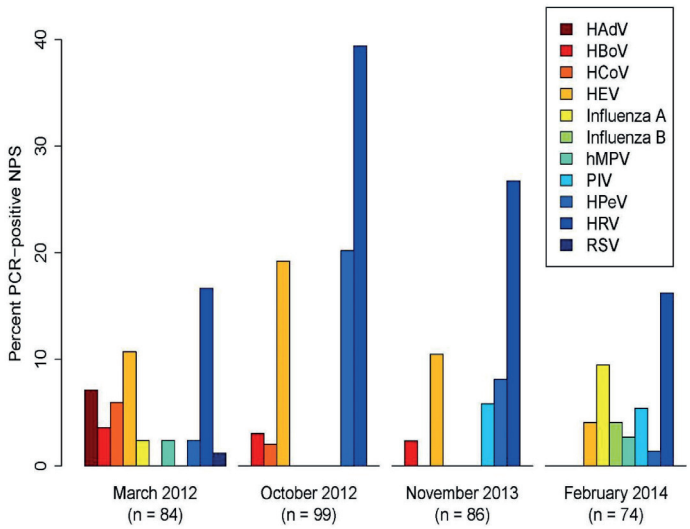

Fig 2. Viral findings at each study visit. Percent nasopharyngeal samples that were positive for each of 11 virus types (genotypes of HCoV and PIV not shown). Nasopharyngeal samples were collected at four different sampling times.

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detected in 88 out of 343 samples (26%), varying from 16% (12/74) to 39% (39/99) at each visit, while HEV was detected in 12% (40/343) and HPeV in 9% (30/343). Ten other viruses were each detected in $\leq$ 3%, including HAdV (n = 6) and HBoV (n = 8), and none was positive for HCoV-OC43, PIV 2 and 3, *Bordetella pertussis*, *Chlamydophila pneumoniae* or *Mycoplasma pneumoniae*.

One virus was detected in 31% (106/343) of the NPS, and two or more viruses were detected in 12% (43/343) (Table A in S1 File). NPS with multiple viruses ($\geq$ 2 viruses) were more frequent than expected if the viruses were randomly and independently distributed among NPS, while single virus samples were less frequent than expected (χ^2 = 21.6, p = 0.0045). Thus, there was a general tendency that viruses occurred together in NPS, although this tendency was not due to the uneven occurrence of viruses among day-care sections and sampling times (Figs 2 and 3, χ^2 = 30.2, p = 0.0020). The co-detection of other viruses appeared in 30 out of 88 HRV-positive samples (34%). The corresponding figures for HEV and HPeV were even higher (23 co-detections out of 40 HEV-positive samples (58%) and 20 co-detections out of 30 HPeV-positive samples (67%)) (Table A in S1 File). HPeV was positively associated with both HEV (χ^2 = 10.7, p = 0.0020) and HRV (χ^2 = 5.4, p = 0.021), while HEV and HRV did not occur more often together than expected by chance (χ^2 = 1.1, p = 0.34). In addition, several of the less frequent virus types, e.g. HBoV, hMPV and PIV, seemed to be positively associated with other viruses (Table B in S1 File).

One or more viruses were detected in 55% (83/152) of the NPS from sections with young children, compared to 35% (66/191) of the samples from older children (p < 0.001). The following virus species were only detected in sections with young children: RSV, PIV-1, hMPV, and HCoV-NL63.

According to the GLMM analysis, the occurrence of HEV-positive NPS varied randomly among combinations of sections and sampling times (Fig 3A); this means that the occurrence of HEV varied from zero to approximately 80% between sections, but it was not the same sections that had a low or high prevalence each time. The probability of HEV-positive NPS decreased with an increasing age of children (z-test: z = -2.4, p = 0.016), and increased with the presence of other viruses (z-test: z = 2.8, p = 0.005). The median age of the HEV positives was 28 months (interquartile range (IQR) 19.3–34.0). Nearly similar results were obtained when modelling the occurrence of HPeV. It varied randomly among sampling times (Fig 3B), decreasing with the increasing age of children (z-test: z = -4.1, p = 0.001), and increasing marginally with the presence of other viruses (z-test: z = 1.7, p = 0.090). The median age of the HPeV positives was 22.5 months (IQR 17.0–30.3). The occurrence of HRV also varied randomly among combinations of sections and sampling times (Fig 3C). There was also a positive effect of the presence of other viruses (z-test: z = 2.0, p = 0.044); however, the presence of HRV was not related to the age of the children (likelihood ratio test: χ^2 = 0.6, df = 1, p = 0.428). Compared to the HEV and HPeV positives, the HRV positives had a higher median age of 35.5 months (IQR 21.0–54.0).

Clinical Findings

In 355 of the 368 inclusions, a clinical examination was performed (96%). NPS were collected from 331 of the examined children, among whom 24% (79/331) had clear findings of a RTI, 41% (135/331) had mild findings and 35% (117/331) had normal findings (Table 1). The baseline characteristics of the children (n = 331) showed that children with a clear RTI were younger and more frequently reported to have more than four upper RTIs per year, compared to those with a mild RTI and no RTI findings (Table 1).

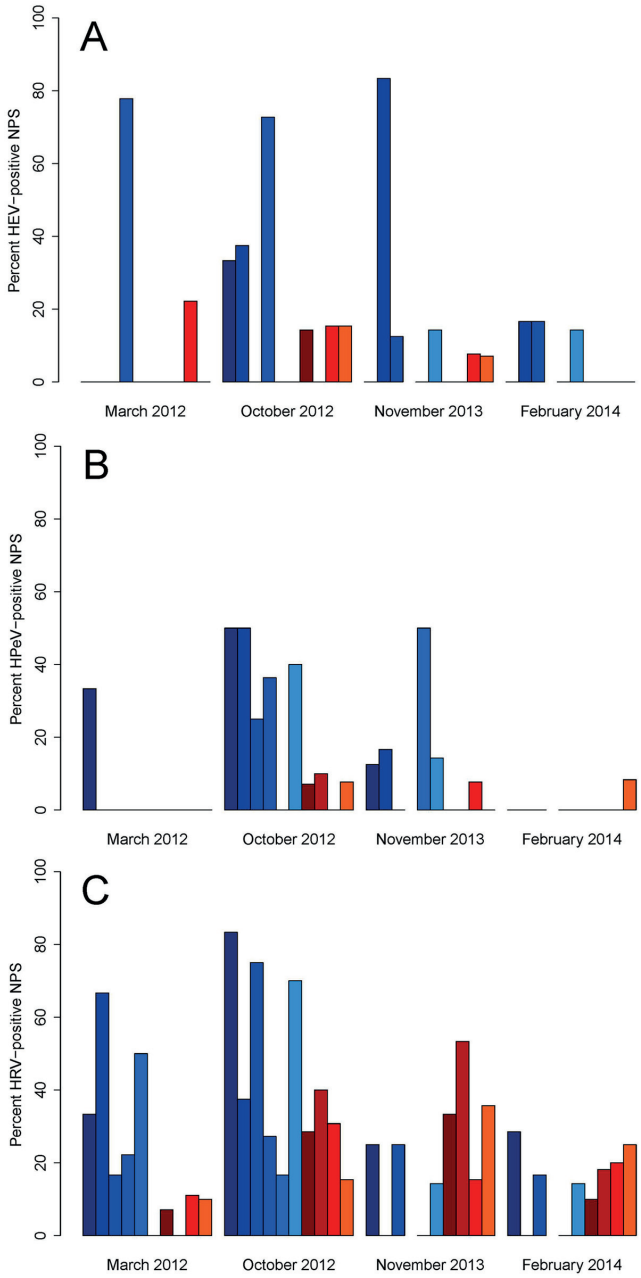

Fig 3. Occurrence of picornaviruses. Percent positive nasopharyngeal samples (NPS) for human enterovirus (HEV) (A), human parechovirus (HPeV) (B) and human rhinovirus (HRV) (C) at four different study visits (sampling times) and in each of 10 day-care sections, six young children sections (blue colors) and four older children sections (red colors). One of the young children sections was not sampled in November 2013 and February 2014.

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Table 1. Baseline characteristics of children at 331 inclusions in the study.

Characteristics	No RTI (n = 117)	Mild RTI (n = 135)	Clear RTI (n = 79)	*P*
Age, month, median (IQR)	51 (41–62)	35 (25–53)	30 (18–47)	<0.001
0–2 years	8 (7)	30 (22)	28 (35)	<0.001**
2–4 years	41 (35)	60 (44)	32 (41)	
4–6.2 years	68 (58)	45 (33)	19 (24)	
Male gender	73 (62)	75 (56)	53 (67)	0.224
Young children section	20 (17)	71 (53)	51 (65)	<0.001***
Older children section	97 (83)	64 (47)	28 (35)	
Parental reports received*	92 (79)	114 (84)	64 (81)	0.489
Premature < 36 GA	11 (12)	8 (7)	4 (6)	0.342
Siblings ≥ 1	79 (86)	83 (73)	42 (66)	0.010
Pets	24 (26)	25 (22)	12 (19)	0.546
≥ 1 parents smoking	9 (10)	11 (10)	7 (11)	0.959
Vaccination	92 (100)	114 (100)	63 (98)	NA
Antibiotic treatment last 6 months	4 (4)	16 (14)	7 (11)	0.068
>4 upper RTI per year	13 (14)	23 (20)	20 (31)	0.034
Allergy	10 (11)	10 (9)	8 (13)	0.722
Asthma	7 (8)	8 (7)	5 (8)	0.977
Eczema	12 (13)	13 (11)	12 (19)	0.382
Epilepsy	0	1 (1)	1 (2)	NA
Heart disease	0	0	1 (2)	NA
Other chronic diseases	1 (1)	1 (1)	0	NA

Data presented as absolute numbers and percent in parenthesis, except from age in months and IQR (interquartile range).

*The number of parental reports received are basis (100%) when calculating percent for all variables except age, gender, Young children section and Older children section. P-values calculated with χ^2 test, except Kruskal-Wallis test for comparing median age.

**Comparing all three age categories.

***Comparing Young children section with Older children section.

GA, gestational age. NA, not applicable. RTI, respiratory tract infection.

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Association between Viral and Clinical Findings

Seventy percent (55/79) of the children with clear signs of a RTI had one or more viruses in the NPS, compared to 41% (55/135) in those with mild findings and 30% (35/117) in those without a RTI ($p < 0.001$) (Table 2). Among the children with a clear RTI and positive NPS, 45% (25/55) were younger than 2 years old (Table 2). HRV was the most frequently detected virus in all clinical groups, varying from 41% (32/79) in the clear RTI group, to 24% (32/135) in the mild group, and 18% (21/117) in children without a RTI ($p = 0.001$) (Table 2). The Ct-values for HRV in NPS were not significantly different between the groups (data not shown). The minimal adequate model from the GLMM analysis of the occurrence of clear findings of a RTI included positive effects of the occurrence of HRV (z-test: $z = 3.0$, $p = 0.002$, and Table 2) and negative effects of children's increasing age (z-test: $z = -3.2$, $p = 0.001$), together with random effects of combinations of day-care sections and sampling times. HEV and HPeV were also detected in all three groups, and most frequently in those with a clear RTI ($p = 0.003$ and 0.005, respectively, Table 2). GLMM analyses of the occurrence of a clear RTI with the presence of HEV or HPeV were inconclusive (data not shown). A few children (n = 12) had influenza viruses A/B, among whom nine had clear signs of a RTI (Table 2). Only 14 children had hMPV (n = 4), RSV (n = 1) or PIV (n = 9), where 11 had mild or clear signs of a RTI. Multiple viruses

Table 2. Viral Findings in 331 inclusions in which children had no, mild or clear findings of a respiratory tract infection (RTI).

Viral findings	Total	No RTI (n = 117)	Mild RTI (n = 135)	Clear RTI (n = 79)	No vs Mild RTI *p*-value	Mild vs Clear RTI *p*-value	No vs Clear RTI *p*-value	All groups *p*-value
Virus negative	186 (56)	82 (70)	80 (59)	24 (30)	0.157*	<0.001*	<0.001*	<0.001*
SV positive	102 (31)	25 (21)	43 (32)	34 (43)				
MV positive	43 (13)	10 (9)	12 (9)	21 (27)				
Positive any virus	145 (44)	35 (30)	55 (41)	55 (70)	0.074	<0.001	<0.001	<0.001
Age 0–2 years	43 (30)	4 (11)	14 (26)	25 (45)	0.016**	0.063**	0.001**	0.001**
Age 2–4 years	53 (36)	11 (31)	26 (47)	16 (29)				
Age 4–6.2 years	49 (34)	20 (57)	15 (27)	14 (25)				
HEV	40 (12)	9 (8)	13 (10)	18 (23)	0.587	0.008	0.003	0.003
HPeV	29 (9)	6 (5)	9 (7)	14 (18)	0.607	0.012	0.004	0.005
HRV	85 (26)	21 (18)	32 (24)	32 (41)	0.264	0.010	<0.001	0.001
HAdV	6 (2)	0 (0)	5 (4)	1 (1)	0.063	0.417	0.403	NA
HBoV	8 (2)	3 (3)	0 (0)	5 (6)	0.099	0.006	0.272	NA
HCoV	7 (2)	3 (3)	3 (2)	1 (1)	1.0	1.0	0.649	NA
Influenza A	9 (3)	2 (2)	1 (1)	6 (8)	0.598	0.011	0.063	NA
Influenza B	3 (1)	0 (0)	0 (0)	3 (4)	NA	0.049	0.064	NA
hMPV	4 (1)	0 (0)	3 (2)	1 (1)	0.251	1.0	0.403	NA
PIV	9 (3)	3 (3)	2 (1)	4 (5)	0.666	0.196	0.443	NA
RSV	1 (0)	0 (0)	1 (1)	0 (0)	1.0	1.0	NA	NA

Data presented as absolute numbers and percentage in parenthesis. P-values using χ^2 test or Fischer's Exact Test. RTI, respiratory tract infection. SV, single virus. MV, multiple viruses, with $\geq$2 viruses. NA, not applicable.

*Comparing Virus negative, SV positive and MV positive.

**Comparing all three age categories.

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were detected in 27% (21/79) of NPS from children with a clear RTI, compared to 9% (12/135) in those with mild and 9% (10/117) with normal findings ($p < 0.001$) (Table 2 and Table C in S1 File).

Parental Reported Symptoms

Based on information collected from the parents, 84% (54/64), 65% (74/113) and 45% (40/89) of the children with clear, mild or no clinical signs of RTI had respiratory symptoms at the examination time or two weeks prior. Among the 55% (49/89) without reported symptoms and with normal findings, still 24% (12/49) had one or more viruses: HCoV-229E (n = 1), HEV (n = 2), HPeV (n = 3) and HRV (n = 8).

Discussion

We detected one or more respiratory viruses in four out of 10 Norwegian children attending day care. All children participated in daily activities, but nevertheless one-fourth had clear signs of an ongoing RTI by clinical examination, and approximately four out of ten had milder signs of RTI. Although those with clear signs had the highest virus detection rate (70%), one-third was still virus positive and without any clinical signs. Hence, our findings indicate that apparently healthy day-care children may harbor respiratory viruses and have clinical signs of

an upper RTI, and even children without clinical signs may be virus positive. It is well-known that young children frequently have symptomatic RTIs, so it is not surprising that children sometimes may also have a RTI with few symptoms when they attend day care [22,23].

Picornaviruses were the most frequently detected viruses during all four sampling times, whereas RSV, influenza virus and other significant pathogens were identified in less than one-fifth of the picornaviruses, and primarily in those with clear RTIs. One out of four visits to the day-care centers occurred during a RSV epidemic, which might explain that only one child had RSV. However, it may be possible that RSV more often causes severe disease and sick leave from day care. Rhinovirus appeared most frequently, but enterovirus and parechovirus were also common. Combinations of day-care sections (younger or older children) and sampling times (seasons) were the most important factors in determining the occurrence of picornaviruses. At any given sampling time, there was a large variation in the frequencies of the three picornaviruses among the various sections, and for most of the sections, there was a large variation at different sampling times. These observations may be relate to the fact that most respiratory viruses are epidemic and easily spread among children who are cared for in separate sections [24]. Indeed, this phenomenon was most common in the sections for the youngest children, who—in particular—are known to challenge good hygiene in day-care centers.

There was a general tendency that viruses occurred together, independent of the influence of sections and sampling times. For instance, the detection of HPeV was associated with both the presence of HEV and HRV, whereas HEV and HRV were related to other viruses, but not to each other. Similarly, others reported that some virus combinations may appear more frequently than others in both children with and without RTI, and that co-infections with viruses may not be random in children with RTI [25–28]. Martin et al. showed that during the progress of a RTI in children, more respiratory viruses may appear [26]. Our data revealed that children with a clear RTI often had frequent upper RTIs during the six months prior to the inclusion in the study, and therefore might have a higher risk of being PCR positive for more than one viruses simultaneously, which is due to possible long-term viral excretion after clinical recovery.

HRV was detected at every sampling time, and was the most common virus. HRV occurred in both the sections of younger and older children, and varied randomly among combinations of sections and sampling times. Children with HRV-positive NPS had increased probability of a clear RTI. Consequently, in this study, HRV was the likely cause of many RTIs in children outside of a hospital, as has been shown by others [22,26,29–31]. However, we also detected HRV in nearly one-fifth of the children without clinical findings of a RTI, while others have detected HRV in asymptomatic children, which is more difficult to explain [9–11,30]. Peltola et al. examined various HRV strains and found that a minor fraction of HRV infections in children may be asymptomatic, and it has also been suggested that HRV PCR tests may persist as positive up to several weeks after clinical recovery [32,33]. Hence, our HRV detection in children without clinical findings can be a result of the carriage of virus after the recovery of symptoms or a newly acquired asymptomatic infection. We found that several children with HRV and clear signs of RTI attended day care and were apparently healthy, which could suggest that HRV in other cases may also cause very mild changes that are hard to detect at all. Recent data have shown that HRV-positive children with and without symptoms developed different systemic immune responses, which support that HRV detection may not always indicate symptomatic HRV infection [34].

HPeV in children has previously been examined only in a few studies, with low detection rates from 1.6% to 2.1% in hospitalized children with RTI, but in a group of asymptomatic young children van den Berg recently detected HPeV in 9% [27,35,36]. Serological studies have documented that most Finnish children may be infected with HPeV1 (83%) and HPeV2 (91%)

before the age of five years [37]. HPeV3 is strongly related to sepsis-like disease and encephalitis, though not RTI, in infants [38]. In the present study, HPeV and HEV were often detected in the same children, attending sections for young children. This co-variation and possible confounding eliminated our possibility to prove that HPeV and HEV were actually related to RTI among the youngest children.

We only detected a few HBoV-positive samples in children with a clear RTI, as well in children who had no signs of RTI, and adenovirus appeared mostly in children with a mild RTI. Recent evidence support that HBoV may cause acute RTI, and adenovirus is a well-known cause of RTI [3,39]. However, it has also been shown that these viruses in particular may sometimes be detected for a long time in the airways, either due to prolonged excretion or due to the re-activation of a latent infection, and all three mechanisms may explain our findings [40,41].

To describe complex microbiology, we collected seasonal samples in both the fall and winter periods in two consecutive years. The day-care section was also considered, and turned out to be an important predictor of virus occurrence. Nasopharyngeal sampling is unpleasant and challenging to perform in apparently healthy children outside health institutions. However, we managed to collect NPS from more than 90% of the inclusions. Ideally, more samples from each child, using a stricter longitudinal design, might have had advantages over the present cross-sectional approach, but in real-life frequent sampling was not possible to attain. A major strength of the study is that pediatricians clinically examined all children, and their findings were used in the classification. Most studies on respiratory viruses and RTIs in day-care settings have relied on parental information of children's symptoms. Nonetheless, we found a poor correlation between symptoms and clinical signs. Others have similarly shown that symptoms are not entirely accurate in predicting an upper RTI in children [42]. On the other hand, the clinical entities of a mild and clear RTI, which were used in our classification, have not been validated. Each study visit was performed three-12 months apart, which is a long time from an epidemic and clinical perspective and, therefore, the analyses were not adjusted for repetitive data.

In conclusion, this study showed that 43% of apparently healthy children attending day care had one or more viruses in NPS, varying from 30% in children with no clinical findings to 70% in those with clear findings of a RTI. Picornaviruses were most frequently detected. Lastly, the viral occurrences were related to age, clinical signs of RTI, location in day care and sampling times (seasons).

Supporting Information

S1 File. Table A. Number virus in nasopharyngeal samples collected at four study visits, and number of nasopharyngeal samples with multiple virus (MV). **Table B**. Virus combinations in 43 nasopharyngeal samples with multiple virus. **Table C**. Virus combinations in 43 nasopharyngeal samples with multiple viruses, in children with clear, mild or no respiratory tract infection (RTI).
(DOCX)

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Author Contributions

Conceived and designed the experiments: NM SAN HD. Performed the experiments: NM SAN LHS SK AS HD. Analyzed the data: NM BP HD. Contributed reagents/materials/analysis tools: NM SAN LHS SK AS HD. Wrote the paper: NM BP SAN LHS SK AS HD.

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S1 file

Table A. Number of viruses in nasopharyngeal samples collected at four study visits, and number of nasopharyngeal samples with multiple viruses (MV).

NPS result	March 12 n = 84	Oct. 12 n = 99	Nov. 13 n = 86	Feb. 14 n = 74	Total n = 343	MV
Virus negative	50 (60)	44 (44)	51 (59)	49 (66)	194 (57)	
Positive any virus	34 (40)	55 (56)	35 (41)	25 (34)	149 (43)	
Single virus	26 (31)	33 (33)	27 (31)	20 (27)	106 (31)	
Multiple viruses	8 (9)	22 (22)	8 (9)	5 (7)	43 (12)	
2 viruses	6 (7)	17 (17)	6 (7)	3 (4)	32 (9)	
≥ 3 viruses	2 (2)	5 (5)	2 (2)	2 (3)	11 (3)	
HAdV	6 (7)	0 (0)	0 (0)	0 (0)	6 (2)	2
HBoV	3 (4)	3 (3)	2 (2)	0 (0)	8 (2)	8
HCoV:	5 (6)	2 (2)	0 (0)	0 (0)	7 (2)	3
HCoV-229E	5 (6)	0 (0)	0 (0)	0 (0)	5 (1)	
HCoV-NL63	0 (0)	2 (2)	0 (0)	0 (0)	2 (1)	
HCoV-OC43	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
HEV	9 (11)	19 (19)	9 (10)	3 (4)	40 (12)	23
Influenza A virus	2 (2)	0 (0)	0 (0)	7 (9)	9 (3)	1
Influenza B virus	0 (0)	0 (0)	0 (0)	3 (4)	3 (1)	1
hMPV	2 (2)	0 (0)	0 (0)	2 (3)	4 (1)	2
HPeV	2 (2)	20 (20)	7 (8)	1 (1)	30 (9)	20
PIV:	0 (0)	0 (0)	5 (6)	4 (5)	9 (3)	8
PIV 1	0 (0)	0 (0)	1 (1)	0 (0)	1 (0)	
PIV 2-3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
PIV 4	0 (0)	0 (0)	4 (5)	4 (5)	8 (2)	
HRV	14 (17)	39 (39)	23 (27)	12 (16)	88 (26)	30
RSV	1 (1)	0 (0)	0 (0)	0 (0)	1 (0)	1

Data presented as absolute numbers and percent in parenthesis, except from absolute numbers of MV.
MV, multiple viruses with ≥ 2 viruses in the nasopharyngeal sample.

Table B. Virus combinations in 43 nasopharyngeal samples with multiple viruses.

	HAdV n=2	HBoV n=8	HCoV n=3	HEV n=23	Infl A n=1	Infl B n=1	hMPV n=2	HPeV n=20	PIV n=8	HRV n=30	RSV n=1
HAdV	-									2	1
HBoV		-	1	3				3	1	6	
HCoV		1	-				1	1		1	
HEV		3		-	1			9	4	13	
Infl A				1	-						
Infl B						-			1	1	
hMPV			1				-			1	
HPeV		3	1	9				-	2	13	
PIV		1		4		1		2	-	5	
HRV	2	6	1	13		1	1	13	5	-	1
RSV	1									1	-

Infl A, influenza A virus. Infl B, influenza B virus. Multiple viruses with ≥ 2 viruses in the nasopharyngeal sample.

Table C. Virus combinations in 43 nasopharyngeal samples with multiple viruses in children with clear, mild or no respiratory tract infection (RTI).

	2 viruses	N	3 viruses	N	4 viruses	N	Total
Clear RTI	All	13	All	7	All	1	21
	HEV, HRV	5	HBoV, HEV, HRV	1	HBoV, HEV, HPeV, HRV	1	
	HEV, HPeV	2	HBoV, HPeV, HRV	1			
	HEV, infl A	1	HCoV, HPeV, HRV	1			
	HPeV, HRV	2	HEV, HPeV, HRV	2			
	HEV, HBoV	1	HEV, HRV, PIV	1			
	HBoV, HRV	1	HRV, infl B, PIV	1			
	HPeV, PIV	1					
Mild RTI	All	10	All	2	All	0	12
	HCoV, hMPV	1	HAdV, HRV,RSV	1			
	HPeV, HRV	2	HEV, HRV, PIV	1			
	HEV, HRV	1					
	HEV, HPeV	3					
	hMPV, HRV	1					
	HEV, PIV	1					
	HAdV, HRV	1					
No RTI	All	9	All	0	All	1	10
	HBoV, HCoV	1			HBoV, HPeV, HRV, PIV	1	
	HPeV, HRV	3					
	HBoV, HRV	1					
	HEV, HPeV	1					
	HEV, HRV	1					
	HEV, PIV	1					
	HRV, PIV	1					

Multiple viruses with ≥ 2 viruses in the nasopharyngeal sample.